DOCTORAL THESIS

TISSUE ENGINEERING FOR PRENATAL APPLICATIONS

Presented by

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Summary

Fetal therapies have become available for a restricted number of life-threatening clinical conditions. Harnessing tissue engineering for prenatal applications has not been widely pursued even though isolating cells from fetal and extraembryonic tissues has been routinely done for years. There are also several studies comparing adult mesenchymal stem cells and their fetal counterparts in repairing various tissues indicating that fetal cells would be more potent for repairing tissue damage and excellent raw material for tissue engineering. Additionally, reconstructing congenital tissue defects such as cleft palate prenatally or in early infantry is expected to produce better results since tissue engineered constructs would be better tolerated and integrated into the recipient owing to more primitive immune system and better healing potential, not to mention that young patients would benefit from living replacement material even more than adults as it would adapt to their growing bodies.

Major reasons for this lack of advances in fetal tissue engineering is that the field of fetal surgery has not been developed enough as well as the fact that any interventions in the uterine cavity poses a significant risk of miscarriage in the form of rupture of the fetal membranes. Iatrogenic preterm prelabour rupture of fetal membranes (iPPROM) occurs in 6-45% of the cases after fetoscopic procedures. It is associated with a range of serious complications including high incidence of morbidity to respiratory distress syndrome (RDS), cerebral palsy, blindness, deafness, or necrotizing enterocolitis posing a significant threat to fetal survival and well-being. Additionally, both fetal and perinatal therapeutic options are often limited due to the lack of sufficient or adequate tissue substitutes for the repair or replacement of defective tissues.

The objectives of this thesis were twofold: i) the development of materials based and tissue engineering based strategies to prevent iPPROM after diagnostic or therapeutic interventions into the amniotic cavity and ii) the generation of tissue substitutes from patient derived fetal cells, which can be employed for prenatal or perinanal transplantation to restore or replace defective tissues.

For the prevention of iPPROM, mussel-mimetic tissue adhesive (mussel glue) a biomaterial which was recently described to not compromise cell viability and to have good tissue sealing capability was compared to fibrin glue. In this *in vivo* study we assessed whether in a mid-gestational rabbit model punctured fetal membranes could be efficiently sealed with mussel glue. In the same study we also assessed whether a scaffold made of decellularized amnion containing the natural biological signals could induce healing once immobilized with glue. Mussel glue showed comparable *in vivo* performance to fibrin glue in sealing fetal membranes though no apparent healing of the membranes could be observed in any of the samples.

The limited ability of naturally derived scaffolds to promote fetal membrane healing inspired the engineering of synthetic plugging material with specifically tailored biological and mechanical properties which could activate the cells in the amnion and induce a healing response. In this thesis the

modularly designed biomimetic poly(ethylene glycol) (PEG)-based hydrogel platform (called TG-PEG from here on) was used together with fetal cells to demonstrate that upon presentation of appropriate biological cues in 3D tissue mimicking environment, mesenchymal progenitor cells from amnion can be mobilized, induced to proliferate and supported in maintaining their native extracellular matrix production, thus creating a suitable environment for healing to take place. These data provide the basis for future engineering of materials with defined mechanical and biochemical properties and the ability to present migration and proliferation inducing factors, namely PDGF, bFGF, or EGF which could be key in resolving the clinical problem of iPPROM and allowing the field of fetal surgery to move forward.

Cleft palate, where the bones of the palatal halves fail to fuse properly, is one of the most common birth defects. Tissue engineering has been envisioned as a treatment option but current approaches have been limited by the lack of i) appropriate autologous cell sources and ii) structural organization and vascularization. Tissue engineering of cleft palates using of autologous amniocentesis-derived and thus ethically unproblematic fetal amniotic fluid cells (AFCs) could be done parallel to the ongoing pregnancy with living reconstruction material being ready when the child is born. We describe using TG-PEG hydrogels to first evaluate 3D osteogenic differentiation of AFCs and creation of vascular structures from AFC derived endothelial cells (enAFC) and undifferentiated AFC either in random or organized channel cocultures *in vitro* and *in vivo*. Next, these approaches were combined in an osteogenic matrix with a channel perfused with enAFC and finally the integration and functional properties of these fetal bone constructs was tested in ectopic mouse model.

In summary this thesis explores using sophisticated biomaterials for guiding cell behavior *in vitro* and *in vivo* in order to tackle clinically relevant prenatal conditions. We think that these results will advance the field of fetal tissue engineering towards clinical reality. Beyond tissue engineering applications physiologically relevant structured tissue mimics would highly desirable for bridging the gap between 2D cell culture and animal trials. The combination of innovative materials with novel technological platforms such as printing, microfluidics, additive or preventive manufacturing provides a great potential to build unprecedented, complex tissue models.

Keywords: Fetal tissue engineering, biomimetic hydrogels, artificial extracellular matrix, fetal membrane repair, amniotic fluid cells, growth factors, bone regeneration, prevascularization, 3D *in vitro* tissue models.

Résumé

Les thérapies fœtales sont disponibles pour un nombre restreint de conditions cliniques où la vie du fœtus est en danger. Le développement de l'ingénierie tissulaire pour des applications prénatales n'a pas été extensivement couvert, alors même que l'isolation de cellules à partir de tissus extra-embryonnaires est une pratique courante depuis de nombreuses années. Il existe également quelques études comparant l'activité réparatrice des cellules souches mésenchymateuses adultes et fœtales. Les résultats indiquent que les cellules fœtales ont un potentiel plus élevé à réparer des tissus endommagés et sont une excellente source de matière première pour l'ingénierie tissulaire. De plus, la reconstruction de défauts congénitaux comme la fente palatine a plus de chances d'aboutir positivement si elle est faite avant ou tôt après la naissance. En effet, l'intégration d'un tissu cellulaire artificiel serait meilleure chez un receveur ayant un système immunitaire primitif et un potentiel de régénération supérieur. Sans mentionner le fait que les jeunes patients bénéficieraient encore plus d'un matériau de remplacement vivant que les adultes dû à l'adaptation de celui-ci au corps en croissance.

Une des raisons pour lesquelles ce domaine d'ingénierie tissulaire fœtale n'est pas à un stade avancé est que la chirurgie fœtale n'est pas suffisamment développée et qu'il existe un risque élevé de fausse couche entraînée par la rupture de la membrane amniotique lors d'interventions intra-utérines. La rupture prématurée pre-partum iatrogène de la membrane amniotique (iPPROM, pour iatrogenic preterm prelabour rupture of fetal membranes) survient dans 6-45% des cas lors d'une fétoscopie. Ces interventions sont associées à une morbidité élevée et à un grand nombre de complications sévères parmi lesquelles le syndrome de détresse respiratoire (RDS, pour respiratory distress syndrome), la paralysie cérébrale, la cécité, la surdité ou encore l'entérocolite nécrotique, menaçant significativement le bien-être et la survie du fœtus. De plus, les options d'interventions fœtales et périnatales sont grandement limitées de par le manque de tissus adéquats pour remplacer ou réparer les tissus endommagés.

Les objectifs de cette thèse sont donc : i) le développement de stratégies basées sur des matériaux et sur des tissus cellulaires pour empêcher l'iPPROM après un diagnostic ou une intervention thérapeutique dans la cavité amniotique et ii) la production de substituts tissulaires à partir de cellules fœtales provenant des patients afin de réparer ou remplacer des tissus défectueux par transplantation pré ou périnatale.

Afin d'empêcher l'iPPROM, une colle semblable à celle produite par les moules (*mussel glue*), qui a été récemment décrite comme non-cytotoxique et ayant une bonne capacité d'obturation, a été comparée à la colle de fibrine. Dans cette étude *in vivo* nous avons évalué si la *mussel glue* pouvait efficacement fermer des membranes perforées sur un modèle de lapin en milieu de gestation. Dans la même étude, nous avons évalué si une structure primaire faite d'amnion décellularisé contenant les signaux biologiques naturels pouvait induire la régénération lorsque cette structure était collée sur la

membrane perforée. La *mussel glue* a montré une performance d'obturation de la membrane amniotique *in vivo* comparable à la colle de fibrine malgré le fait qu'aucune régénération flagrante n'ait été observée dans les échantillons.

Cette aptitude limitée qu'ont les structures primaires décellularisées à promouvoir la régénération de la membrane amniotique a poussé au développement d'un matériau implantable pouvant être modifié pour contenir spécifiquement des propriétés biologiques et mécaniques afin d'activer les cellules amniotiques et induire une réponse régénératrice. Dans cette thèse, la plateforme modulaire d'hydrogel à base de poly(ethylene glycol) (PEG) (nommé TG-PEG à partir d'ici) a été utilisée en combinaison avec les cellules fœtales pour démontrer que les cellules progénitrices mésenchymateuses pouvaient être mobilisées, poussées à la prolifération ainsi qu'à la production de matrice extra-cellulaire à condition que les signaux appropriés aient été présentés dans un environnement simulant le tissu en 3D. Tout ceci afin de créer un environnement propice à la régénération. Ces données fournissent la base pour le développement futur de matériaux ayant des propriétés mécaniques et biochimiques définies ainsi que la capacité de présenter des facteurs provoquant la migration et la prolifération, en l'occurrence PDGF, bFGF, ou EGF. Ceci pourrait être la clé pour résoudre le problème clinique qu'est l'iPPROM et pour stimuler l'innovation dans le domaine de la chirurgie fœtale.

La fente palatine, résultant de la non-fusion des deux os de chaque moitié du palais, est le défaut le plus commun à la naissance. L'ingénierie tissulaire serait une option de choix pour le traitement de ce problème mais cette approche est limitée par le manque i) de source de cellules autologues appropriées et ii) d'organisation structurelle et de vascularisation. L'ingénierie tissulaire de la fente palatine utilisant des cellules autologues du liquide amniotique (AFCs, pour amniotic fluid cells) provenant d'amniocenthèses, et donc éthiquement non-questionnable, pourrait être faite en parallèle à la grossesse afin d'avoir un tissu vivant de reconstruction prêt pour la naissance de l'enfant. Nous décrivons à l'aide d'hydrogels de TG-PEG la différenciation ostéogénique d'AFCS en 3D et la création de structures vasculaires à partir de cellules endothéliales dérivées d'AFC (enAFC) ainsi qu'à partir d'AFC non-différenciées, d'une part en co-cultures désordonnées ou organisées en canaux *in vivo* et *in vitro.* Ensuite, ces approches ont été combinées dans une matrice ostéogénique contenant un canal perfusé avec des enAFC. Et pour finir, l'intégration et les propriétés fonctionnelles de cette structure fœtale osseuse ont été testées dans un modèle ectopique sur la souris.

En résumé, cette thèse explore la possibilité d'utiliser des biomatériaux sophistiqués guidant le comportement cellulaire *in vitro* et *in vivo* afin d'aborder certains problèmes prénataux pertinents. Nous pensons qu'avec ces résultats, nous allons faire avancer le domaine de l'ingénierie tissulaire fœtale vers la réalité clinique. Au-delà des applications d'ingénierie tissulaire, des structures pertinentes physiologiquement imitant des tissus seraient très souhaitables afin de combler l'espace entre les cultures en 2D et les expérimentations animales. La combinaison entre matériaux innovants

avec de nouvelles plateformes technologiques comme l'impression, la micro-fluidique, la manufacture additive ou préventive fournit un grand potentiel pour la construction de modèles tissulaires sans précédents.

Mots clés: Ingénierie tissulaire foetale, hydrogels biomimétique, matrice extra-cellulaire artificielle, réparation de la membrane amniotique, cellules du fluide amniotique, facteurs de croissance, régénération osseuse, prévascularisation, modèles de tissus en 3D

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CHAPTER 1 Introduction

Adapted from

Recent Advances in 3D Tissue Models

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MOTIVATION

Tissue engineering attempts to repair or replace damaged tissues by using biomaterials and/or (stem)cells. Since its birth the field has addressed almost all tissues found in the body but mainly focused on mature tissues in adults. Applying tissue engineering prenatally on a still developing human body has been touched upon but has not been widely pursued. This seems illogical since tissue engineered constructs would be better tolerated and integrated into young recipients owing to their more primitive immune system and better healing potential, not to mention that young patients would benefit from living replacement material even more than adults as it would adapt to their growing bodies. Major reasons for this lack of advances in fetal tissue engineering are that the field of fetal surgery has not been developed enough as well as the fact that any interventions in the uterine cavity pose a significant risk of miscarriage in the form of rupture of the fetal membranes [1]. Iatrogenic preterm prelabor rupture of fetal membranes (iPPROM) has been associated with a range of serious complications including high incidence of morbidity to respiratory distress syndrome (RDS), cerebral palsy, blindness, deafness, or necrotizing enterocolitis[2].

Fetal tissue engineering in utero or after birth has been suggested for conditions such as spina bifida [3, 4], congenital muscular defects such as congenital diaghpragmatic hernia [5-7], skin [8], bladder and abdominal wall defects [5, 9], cardiac anomalies [10] and bony defects such as cleft palate [11] as well as for dealing with fetal membrane defects during pregnancy [12, 13]. Most suitable cell source for engineering fetal tissues would naturally be the fetus itself or more practically amniotic fluid or other extraembryonic tissue containing fetal cells. While the isolation of cells from extraembryonic tissues such as placenta [14], fetal membranes [15] and umbilical cord [16] right after birth is relatively easy and without ethical concerns there is a significant risk of contamination and the engineered tissues will not be available at birth. In contrast, an interesting aspect of harvesting cells from amniotic fluid or the fetus is that it is possible to prepare autologous tissue engineered replacement material parallel to the ongoing pregnancy. Reconstruction using this material can then be performed later in the pregnancy or more feasibly after birth. In addition to this it is possible to isolate cells of fetal origin from extraembryonic tissues

There are several studies comparing adult mesenchymal stem cells with their fetal counterparts for repairing various tissues [17, 18], all pointing to the superiority of fetal cells. They grow faster [19-21], are described to be more primitive [19, 22], are more resistant to hypoxia [20, 23] and produce more and better quality (tissue specific proteins in stead of scar tissue) extracellular matrix [17, 21, 24, 25] making them more potent for repair of tissue damage and excellent raw material for tissue engineering.

To take full advantage of these cell sources for constructing functional living replacement materials, highly sophisticated scaffold materials that allow the three dimensional structuring of cells, matrix

components and cell-instructive cues are needed. Modern synthetic hydrogels are designed to provide an empty canvas on which biological functionality can be added in a controlled fashion resulting in extracellular matrix mimicking environments. In this thesis both iPPROM and treating congenital malformations are addressed using a tissue engineering approach. First two chapters descripe novel approaches for sealing and healing fetal membrane defects. The third chapter tackles using amniotic fluid cells as an autologous source for producing prevascularized bone constructs for reconstruction material for cleft palate and other congenital malformations affecting the skeleton. Since each chapter provides an introctuction to the specific topic in it, the next part of this introduction chapter focuses on discussing advances in hydrogel biomaterials, such as the so called TG-PEG platform [26] used in this thesis, and their manipulation which provides the technological basis for the later applications in engineering fetal tissues**.**

BACKGROUND

Physiologically relevant tissue models that bridge the gap between 2D tissue culture and animal trials would be highly desirable to study the function of tissues in health and disease as well as for the validation of lead compounds during drug development. The field has made impressive advances in 3D culturing cells and organoids in naturally derived materials. Novel, rationally designed, biomimetic materials have been established, which allow the almost individual variation of matrix parameters, such as stiffness, cell adhesion, degradability, or growth factor binding and controlled release. The combination of innovative materials with novel technological platforms such as printing, microfluidics, additive or preventive manufacturing provides a great potential to build unprecedented, complex tissue models. Here we review recent advances in the design of materials building blocks which allow the formation of 3D structured microenvironments. The next part of this chapter focuses on hydrogel materials for tissue engineering and on strategies to locally position cell-instructive molecular cues. Also models which would allow the investigator to controllably manipulate cells in their 3D context with the aim to generate complex but yet scalable tissue models are discussed.

Why culture cells in 3D?

For the understanding of fundamental biological phenomena and for development of novel drugs there is a large need for highly reproducible, reliable, and physiologically relevant test platforms. Over the past decades a wealth of detailed insight into cellular and molecular details has been acquired using 2D cell culture models. This knowledge has frequently been transferred to animal models, where among other techniques, genetic tools have helped to understand individual factors in the complex systemic context. However, though in vitro and in vivo experiments have been a very successful combination for many decades, both of them have clearly indefinable limitations. 2D tissue culture models provide limited information regarding physiologically relevant i) tissue morphogenesis ii) chemotaxis and

haptotaxis iii) cell morphology iv) matrix remodeling and v) effects of matrix mediated signaling. In contrast, in vivo animal models are limited by i) throughput ii) systemic effects and compensation phenomenon, iii) differences in physiology between species, and iv) ethical concerns.

In order to study cells in a physiologically relevant environment, conditions nearly identical to the ones of the native tissue with respect to composition and 3D arrangement would have to be established in vitro. Cells in tissues are embedded in a microenvironment consisting of neighboring cells, extracellular matrix components and signaling molecules. Of course the cellular response is the result of an integration of these various signals. By dynamic changes of the microenvironmental composition, the cellular response can substantially adapt during both development and healing. To recapitulate developmental, physiological, or even pathological situations, ideally cells, matrix components and signaling cues could be arranged in a rational and three dimensionally controlled manner, such that single parameters can be individually varied.

In this chapter we will commence with a short discussion of 3D cell culture models which are based on matrix free approaches. Since such assays are performed at high cellular density and rely on cell´s own matrix production, the additional encapsulation of cells in a provisional matrix clearly offers the opportunity to provide and vary matrix signals. Thus, a summary of achievements using biologically derived materials to generate in vitro tissue models will be given next. Although with scaffold based approaches impressive advances have been achieved, we will here focus our discussion mainly on hydrogel systems. For the establishment of fully defined, engineered tissue models the precise control over all components and their exact positioning in 3D would be desirable. Great advances in the engineering of naturally occurring and biomimetic extracellular hydrogel matrices towards the control of biological functions have been made. We will describe their fundamental design principles of currently available hydrogel platforms. Having done so, the achievements in design of functional building blocks, their spatial and temporal arrangement and release will be discussed. Finally, examples of sophisticated 3D tissue models which will be the basis for the development of in vitro tissue homologues will be given.

2D cell cultures

Many cell types are adhesion dependent and cannot be grown in suspension cultures without mechanical support. All freshly isolated, culture expanded as well as immortalized cells have for many decades been cultured on tissue culture polystyrene plastic. In such cultures cells normally spread, form focal adhesions and stress-fibers throughout the cytoplasm. Such cultures can relatively easily be used to determine gene and protein expression, biochemical pathways, intracellular trafficking just to mention a few. However, 2D cultures do only to a certain degree represent the physiological environment with respect to cell shape, cell-matrix, cell-cell interactions, local chemotactic and haptotactic gradients, nutritional status, or interstitial flow [27, 28]. For example, tumor cells, when grown on 2D substrates are flat, whereas in 3D they adapt a round morphology, much like seen in

cancer biopsies. Also mesenchymal cells independent of substrate composition, exhibit bipolar spindle shaped morphology in 3D as compared to the observed artificial dorsal (upper side) ventral (lower side) polarity in 2D [29] as here illustrated for cells being cultured on top (Figure 1.1A) or inside (Figure 1.1B) poly(ethylene glycol) (PEG) hydrogels of identical composition.

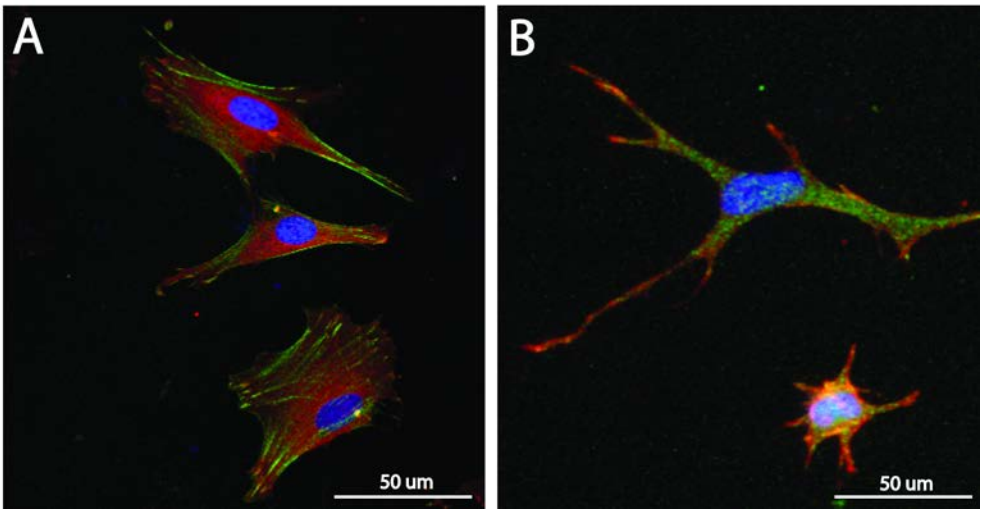

Figure 1.1 Morphology of murine fibroblast cultured A) on 2D substrates and B) in 3D cultures.

Not surprisingly, recent findings suggest a strong correlation between cell shape and function. Impressively, the changes in morphology and cell-substrate interactions in 3D cultures translate to differential cell signaling [27] resulting among others functional differences in reduced sensitivity of tumor cells towards radiotherapy (cell adhesion-mediated radioresistance) and chemotherapy (cell adhesion-mediated drug resistance) [30]. The decreased sensitivity to doxorubicin or etoposide observed in small cell lung cancer cells cultured on fibronectin or laminin [31] and the increased radiosensitivity of lung carcinoma cells with altered cell shapes by destabilization of the actin filaments [32] point towards effects mediated by both matrix components as well as the 3D arrangement. Furthermore in many organs and tissues, different cell-types are arranged in spatially defined, repetitive manner and are structurally integrated with their neighbors. Together this might explain why some of the effects seen in 2D cultures are significantly different in the native tissue and thus findings from 2D cultures and even uniform 3D cultures likely have a limited predictive value [33]. Thus, the current workflow in pharmaceutical industry consists of screenings in 2D followed by validation in simple 3D models and animal models.

However, due to species specific differences in physiology, metabolic activity and cell function, also animal models have a restricted predictive value regarding drug therapeutic response. Thus, 3D tissue and organoid models partially representing functional properties of physiological or pathological human tissues are now seen as an interesting approach to bridge the gap between traditional 2D cultures and animal models [28, 34].

Top down approaches (matrix free)

Typically tissue extracellular matrix (ECM) undergoes tightly regulated remodeling through cells continuously secreting and subsequently remodeling their own ECM consisting of molecules such as collagens, fibronectin, elastin, and proteoglycans [35]. These secreted tissue specific ECM components in turn will be sensed by cells integrin receptors and eventually influence the behavior of the cell itself as well as the one of neighboring or invading cell. When taken in culture, cells produce their own ECM [36] and are structurally and functionally integrated with their neighboring cells and the ECM [33]. During cell passaging, in order to permit the transfer of tightly adherent cells from one substrate to the next, cell-substrate interactions need to be disrupted by treatment with trypsin and EDTA by proteolytic cleavage and destabilizing of protein interactions. Due to cell surface located proteins (e.g. growth factor receptors, syndecanes, integrins, proteases and their regulators) engaged in receiving signals form the environment and providing modifying signals to the environment, passaging can have a significant impact on cell behavior.

Microtissues

Microtissues, also called spheroids, are 100 to 500 µm sized multicellular clusters which can be formed on non-adhesive substrates by hanging drops, or spinner flasks [37]. Microtissues can be assembled without the addition of matrix components and allow the formation of multicellular structures without the need of external signals. Additionally, within them cell-cell connections and connections of cells with their own ECM can be established. Due to 3D arrangement, cells adapt a more natural morphology and structure and function of deposited ECM takes the role of the natural environment. Microtissues present a straight forward approach to assemble a large variety of cell types as monotypic cultures or as co-cultures in a mostly natural environment, thus they have a great potential to be used in scalable drug screening platforms [38, 39]. They allow the determination of biochemical, morphological, functional and morphogenesis related parameters.

The major limitations of microtissues are the heterogeneity of the cellular environment and the limited ability to control the initial patterning of cells. The heterogeneity of cells within microtissues is largely due to the non-homogeneous distribution of nutrients and oxygen, resulting in a hypoxic core with apoptotic cells and sufficiently nourished cells located on the outside. The difficulty to control the spatial arrangement of multiple cell types, could potentially be overcome by varying of the microtissue formation. The value of microtissues will rather be in understanding tissue self-organization processes and contact-mediated interactions between different cell types. For example in an attempt to create functional myocardial tissue rat cardiomyocyte based microtissues were formed and exhibited coordinated beating after electrochemical coupling. When microtissues were coated with endothelial cells and assembled into macrotissues of mm^3 scale, they spontaneously formed microvessels with native vessel ultra-structural morphology. Transplantation of such prevascularized tissues into chick

embryos or rat pericardium demonstrated both the functional integration of microvessels (60 hours post transplantation) and the co-alignment of transplanted and host cardiomyocytes [40].

In order to take control over positioning of individual cell-types DNA-programmed assembly of microtissues has been employed [33]. In order to take control over positioning of individual cell-types, DNA-programmed assembly of microtissues has been employed [33]. Using this approach the assembly of microsphere consisting of one MCF10A mammary epithelial cell with activated H-Ras and multiple control MCF10A cells could be achieved. The generated cell-to-cell variability in signaling was shown to lead cell mobility which is due to heterogeneity in pathway activation rather than in absolute pathway activity. Alternatively, polydimethylsiloxane (PDMS) molds have been generated in which by sequential seeding and sedimentation cells could be precisely positioned in 3D [41]. Such arrangement together with the stabilization of the structures within a hydrogel allowed the formation of liver-mimicking tissue structures in vitro. The arrangement of hepatic aggregates and liver endothelial cells demonstrated that the geometry of arrangement can have a large influence on cell function. If hepatocytes and endothelial cells were positioned in a compartmentally distinct localization allowing only paracrine signaling, albumin production could be sustainably enhanced compared to juxtaposed position. As such complex assays are clearly needed for the evaluation of tissue functions, such as that of liver, the combination of principles from bottom-up and top-down approaches as well as the use of patients own cells will be necessary.

Cell sheet engineering

An interesting alternative to microtissues is cell-sheet engineering. This technology relies on a purely cell based assembly of tissue structures. Again, cells are allowed to establish their own ECM and to form mature tissue structures. One of the first examples using this approach was the assembly of intermediate size blood vessels (3mm inner diameter) [42]. The construction of the vessel was initiated by first culturing fibroblast cell sheets, which were wrapped around a support and formed a tubular tissue. After decellularizing the remaining tissue matrix, forming an inner membrane, it was mounted on a perforated tubular mandrel before a sheet of smooth muscle cells was wrapped around to form the vascular media. This construct was maturated in a bioreactor before an additional sheet of fibroblasts, the adventitial layer, was wrapped around the vascular media. After another period of maturation in a perfusion bioreactor the tubular construct was removed from its support and seeded with endothelial cells which again were allowed to grow and maturate for one week. Histologically the constructs resembled a native vessel with intima, media, and adventitia and due to established ECM they were shown to resist physiologically relevant pressures (larger than 2000 mmHg). Vessel constructs similarly produced using fibroblasts and endothelial cells were implanted as arteriovenous shunts in end-stage renal disease patients. In this study, the tissue engineered constructs showed a

patency rate of 78% after one month and 60% after 6 month of transplantation. Additionally, the constructs showed impressive resistance to intimal thickening and aneurysm formation [43].

Recent efforts towards the construction of engineered vessels concentrated on the development of production methods which give rise to constructs with better mechanical properties, are less time consuming, and less dependent on cells' capacity to produce ECM. These aims were achieved by employing a single step assembly protocol [44] or the formation of fibroblast derived, decellularized ECM which could be seeded with smooth muscle cells [45]. Together with the engineering of the vascular adventitia containing vasa vasorum, which was shown to improve graft integration and inosculation, engineered blood vessels hold great promise to become clinically applicable tissue engineered products [46].

An alternative approach to harvesting of contiguous sheet of cells has been pursued by the development of surfaces where a simple shift of temperature (Reviewed in [47]) or local charge [48] leads to a change of surface hydrophilicity or disintegration of the surface coating. The resulting sheading of the cell sheets without the use of proteolytic enzymes and EDTA allows cells to retain their structural and functional properties and to remain within the intact and functional ECM throughout the transfer [49]. The potential advantage of cell-sheet engineering lies in the ability to generate tissue constructs, which can be highly structurally ordered and allow the use of multiple different cell types. Initial attempts however have relied in single cell type sheets, which have even translated to clinical applications such as for the replacement of corneal tissue. Stratified epithelial cell sheets with normal cell profiles and functions were produced in human autologous serum and in absence of feeder layers or bacterial or animal derived products [50]. Later, using microcontact printing of fibronectin onto thermoresponsive surfaces, patterned cell sheets containing structurally arranged endothelial cells and hepatocytes could be produced [51]. In order to treat infarcted hearts, multiple myocardial cell sheets were stacked on top of a perfused vascular bed to generate functional 3D myocardial tissue constructs which contain a perfused vascular network [52]. With the advances in cell deployment using dispensing robots or microfluidics as well as the layer-by-layer assembly of cell sheets could clearly help to generate more complex 3D tissue models. More likely, the integration of matrix free and matrix based approaches will be needed to generate models which recapitulate spatiotemporally regulated processes as they occur during tissue morphogenesis and healing.

Bottom up approaches (cells in biomaterials)

In classical tissue engineering applications cells have been used in combination with biomaterials [53] which, with the aim to reconstruct or heal tissues, nowadays are often complemented with cell instructive factors provided with the biomaterial or with the cell culture medium. Biomaterials in this context are meant to substitute the native ECM and thus provide cells with an adequate provisional environment during tissue formation. ECMs in naturally occurring tissues consist mainly of fibrous proteins (collagens, fibrinogen, elastin, laminins) and proteoglycans. Both classes of molecules

contribute to the mechanical (tensile and compressive) properties of tissues and are additionally involved locally in providing adhesion sites and molecular cues to embedded cells. Tissue engineering aims at replacing this naturally occurring matrix with (in most attempts) a provisional one to provide a template for the formation of novel tissue structures. A myriad of literature can be found on ceramic, polymeric, or biological materials which by different manufacturing processes give rise scaffolds with variable porosity, pore size distribution, and interconnectivity [54]. By providing structural support and a basis for the deposition of cell´s own ECM, such porous scaffolds provide great platforms for both in vivo healing strategies and for the culture of cellular constructs in vitro. However, since the colonization with cells relies on their invasion from the outside or on dynamic seeding, porous scaffolds are not amenable for the exact positioning of cells as would be needed for advanced in vitro 3D tissue models. Therefore, within this chapter we will concentrate on hydrogel materials which are the generally used platform in advanced tissue models today.

Hydrogels

Hydrogels, comparable to glycosaminoglycans of the native ECM, are hydrophilic polymer networks which are highly swollen in aqueous solutions as they imbibe large quantities (often up to 99%) of water [55]. Hydrogels, additionally to having similar mechanical properties to ECMs permit the efficient diffusion of respiratory gasses, nutrients or waste products and signaling molecules. Thus, they are often considered as good ECM models and broadly used for tissue engineering applications [55]. In all of these systems functional elements and means to control the materials function have been introduced over the years moving from simple hydrogels to hydrogels with more advanced properties. Functional elements can often be employed in different materials and thus synthetic and biological elements become more and more integrated. However, in order to structure the following sections we have made the distinction between biologically derived and synthetic materials.

Biologically derived hydrogel materials

In early tissue engineering applications cells have been encapsulated and 3D-cultured in a large variety naturally occurring, hydrogel forming, protein based materials such as collagens, fibrin, matrigel, or sugar based materials such as alginate, agarose, hyaluronic acid, and chitosan [56]. Such 3D-cultures have enabled the formation of relatively simple skin, bone, cartilage tissue models, to name a few. Additionally they have allowed the study of morphogenetic events of for example intestine or mammary gland under tightly controlled culture conditions (Reviewed in [57, 58]). They have also highlighted the influence of the dimensionality on the outcome of biochemical parameters. Due to the supportive biological properties of the naturally derived materials (e.g. their bio-degradability and presentation of integrin-ligands) such early approaches have led to impressive advances in the engineering of advanced tissue models.

Matrigel

Matrigel™ is a matrix that mainly consists of laminin, type IV collagen, entactin, heparin sulfate proteoglycans [59]. Since Matrigel™ is isolated from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, its composition is relatively ill defined, has large batch-to-batch variation, and can contain tumor derived proteolytic enzymes and growth factors which can promote cellular function in an unpredictable manner. Despite the limitations of Matrigel™, this system has been successfully employed in a myriad of both tissue engineering applications and the formation of organoids in vitro. Impressive examples are the formation of small intestine organoids starting from intestine biopsies or even single intestine derived leucine-rich-repeat-containing G-protein-coupled receptor 5 (Lgr5) positive intestinal crypt stem cells [60].

Collagen and gelatin

(Review on collagen in [61]) Collagens are the most abundant proteins in the ECM of most tissues. Besides providing tensile strength to the tissue, by presentation of integrin binding sites in their native and proteolytically degraded form they largely contribute to cell function and signaling. Due to their limited solubility and the presence of covalent intermolecular (Schiff base, aldimine) cross-links in native tissues, collagens are commonly isolated using pepsin or acidic extraction conditions. Thermal denaturation of insoluble collagen results in gelatin, which dependent on the source can have variable properties.

Physical hydrogels formed by collagen type I provide excellent cell substrates both in vitro and in vivo. For example, plastic compressed collagen type I hydrogels have been used to engineer dermo-epidermal skin substitutes that can be formed at clinically relevant sized and be transplanted and in a rat model successfully reconstitute full-thickness skin defects [62]. However, as during the common isolation process of collagens the Schiff base is reversed to amines and aldehydes which further are converted to alcohols collagen cross-links cannot spontaneously form [63]. Thus in order to improve collagen and gelatin stability, cross-linking using glutaraldehyde or carbodiimide is needed, which is clearly not applicable for the delivery of proteins or encapsulation of cells.

Fibrin

Fibrin gels are formed by the thrombin mediated cleavage or fibrinogen resulting in the release of the fibrinopeptides and the lateral aggregation of fibrin monomers to fibrin fibrils. This physical matrix is subsequently enzymatically cross-linked by the transglutaminase factor XIIIa (FXIIIa). Fibrin hydrogels are commonly used as surgical tissue glue. As they are highly biocompatible they have in large number of in vitro culture cell and in vivo tissue applications led to impressive results. For example, fibrin in combination with knitted fabric has been used to create myocardial patches. Upon in vitro culture under cyclical stretch the provisional matrix was remodeled as shown by the increasing amounts of

collagen after one week of culture. After subcutaneous implantation in rats cardiomyocyte survival and vessel ingrowth into these constructs was shown [64].

Alginate

Alginate is an unbranched, sugar based material which consists of 1-4´-linked b-D-mannuronic acid (M) and a-L-glucuronic acid (G) derived from brown algae. Alginate hydrogels readily form by the cooperative binding of Ca^{2+} ions to the G-block [65]. By the association of two G-blocks, junctions are being formed, leading to the formation of a network structure. Alginate hydrogels are highly biocompatible but are largely devoid of biological function in mammalian tissues. Thus for tissue engineering applications they need the chemical integration of adhesion sites such as RGD [66]. Additionally, the loss of divalent cations by diffusion results in an uncontrolled disintegration of the hydrogels, which can be controlled by oxidation and covalent cross-linking [56]. Modified alginate hydrogels have indeed been shown to be very suitable for the delivery growth factors as well as tissue engineering applications. For review please refer to [67].

Hyaluronic acid

Hyaluronic acid (HA) is a negatively charged matrix component which is present in the ECM of most tissues [68] and contributes to the compressive properties of tissues [69]. It is a linear polysaccharide of 100 to 8000kDa, consisting of repeating disaccharides of -1,4-D-glucuronic acid-b-1,3-N-acteyl-D-glucosamine. HA is not immunogenic and can easily be chemically modified [70]. Due to binding to CD44 HA is involved in many cellular processes [71]. Therefore HA is also often combined and cross-linked with synthetic polymers to form semisynthetic hydrogels for protein and cell delivery. For review please refer to [72].

Engineering of naturally derived biomaterials

However, the major drawback in the use of naturally occurring materials in engineering applications is their inherent biological properties such as the presentation of integrin ligands, the proteolytic degradability, and available specific and unspecific protein binding sites. To achieve prolonged materials stability, collagen hydrogels have been crosslinked by N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) [73]. To decrease plasmin digestion of fibrin hydrogels, aprotinin was engineered to be covalently incorporated in fibrin [74]. Additionally, the chemical or enzymatic coupling of functional groups such as heparin or growth factor binding peptides to naturally occurring hydrogels have allowed the mimicking of naturally occurring ECM growth factor binding (Figure 1.2A) [74-79]. The covalent enzymatic or chemical immobilization of engineered growth factors or the use of affinity linkers have provided an another possibility to generate growth factor repositories for the sustained long term delivery of minute quantities of highly potent growth factors such as vascular endothelial growth factor (VEGF), bone morphogenetic proteins (BMPs), or insulin like growth factor

(IGF) (Figure 1.2B) [80-82]. The various strategies employed to immobilize growth factors in biological biomaterials are summarized in Table 1.1.

Table 1.1: Strategies for immobilization and release of growth factors in naturally occurring hydrogels (adapted from [83]).

Matrix backbone	Linker	Ligand				Ref.
		Native form	Modification	Immobilization	Release from Matrix	
Fibrin	heparin, Gln-HBP[1]	bFGF, β-NGF, PDGF-BB, NT-3	-	affinity	enz.	dissoc. [77, 84-91]
Collagen	heparin, EDC/NHS[1]	VEGF, bFGF, SDF-1α	-	affinity	enz.	dissoc. [92-98]
Fibrin	FN-III9-10/12-14-Gln[1]	VEGF, PDGF-BB, BMP	-	affinity	enz.	dissoc. [79, 99]
Fibrin	-	bFGF	Ligand-Kringle fusion	affinity	enz. / dissoc.	- [100, 101]
Collagen	-	PDGF-BB, EGF, BDNF	Ligand-CBD fusion	affinity	enz. / dissoc.	- [102-104]
Collagen	SG-PEG-SG[1, 2]	TGF-ß2	chemical	covalent	enz.	- [105]
Collagen	SS-PEG-SS[1, 2]	$VEGF_{165}$	chemical	covalent	enz.	- [106]
Collagen	EDC/sulfo-NHS[1, 2]	$VEGF_{165}$	chemical	covalent	enz.	- [107]
Fibrin	SMCC-Gln[1, 2]	KGF	chemical	covalent	enz.	- [108]
Fibrin	BTC-PEG-BTC[1, 2]	SDF-1α	chemical	covalent	enz.	- [109]
Fibrin	-	β-NGF, BMP, $VEGF_{121}$, ΔAng-1 ephrin-B2, PTH, L1Ig6, IGF-1 Aprotinin	Ligand-Gln fusion[1]	covalent	enz.	- [80-82, 110-119]
Fibrin	Gln-PIGF(123-144)	VEGF, BMP-2, PDGF-BB, multible other ligands	-	affinity	enz.	dissoc. [120]

[1] reacting with matrix; [2] reacting with Ligand;

Abbreviations: enz., enzymatic; dissoc., dissociation; Gln, NQEQVSPL-peptide; Gln-HBP, bifunctional NQEQVSPL-heparin binding peptide; NHS, N-hydroxysuccinimide; EDC, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide; FN-III9-10/12-14, pieced together Fibronectin III fragments from the 9^{th} to 10^{th} and 12^{th} to 14^{th} repeats; SG-PEG-SG, disuccinimidyldiglutarate-polyethyleneglycol; SS-PEG-SS, Disuccinimidyldisuccinatepolyethyleneglycol; SMCC, Succinimidyl trans-4-(maleimidylmethyl) cyclohexane-1-carboxylate; CBD, collagen binding domain; ΔAng-1, truncated version of Angiopoietin-1; PIGF(123-144), placental growth factor derived peptide sequence amino acids 123-144.

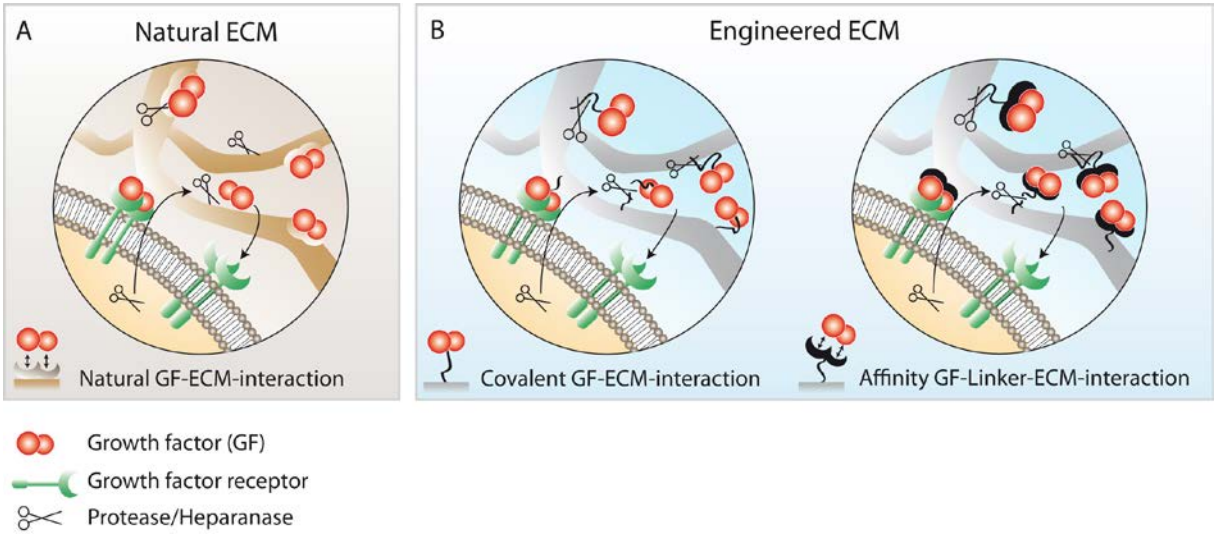

Figure 1.2 Growth factor immobilization and release in A) natural ECM, B) engineered ECM with covalent interactions and C) with affinity linkers.

All mentioned growth factor presenting materials can be engineered to instruct encapsulated or recruited cells to undergo differentiation or induce morphogenic processes in vitro and in vivo. For example the covalent immobilization of VEGF generated a growth factor repository for the sustained long term delivery of minute quantities the highly potent growth factor in an active form [121]. Indeed this strategy has enabled the induction of large numbers of patent and morphological normal blood vessels in small animal models [111, 122]. Similarly, recently described linker peptides derived from fibronectin (FN) [79] have been shown to mediate the retention of growth factors and cytokines within fibrin hydrogels. Additionally to the efficient delivery of factors by the FN it also coordinated growth factor receptors and integrin signaling. Delivery of platelet derived growth factor-BB (PDGF-BB) was shown to improve in vitro mobilization of smooth muscle cells and in presence of BMP-2 to improve bone healing in vivo.

Above examples illustrate that major achievements made by the engineering of biologically derived materials. However, though some of the inherent properties of biological materials can be overcome with engineering strategies, others such as proteolytic stability, presentation of integrin ligands, low affinity binding of proteins, or gelation kinetic cannot be so easily manipulated.

Synthetic hydrogel matrices

Ideally, for engineering applications the precise and independent control over following hydrogel parameters would be available: mechanical properties, gelation kinetics, micro and/or nanoarchitecture, presentation of adhesion ligands, proteolytic degradability, specific protein binding and release. All of these parameters have to be considered when designing tissue engineering applications in vitro. Furthermore, mimicking dynamic changes throughout morphogenesis, materials

should be available which allow trigger inducible change in materials properties. Some of these materials have successfully been engineered to bind and locally present biological cues.

Backbone design

Multiple (cationic, anionic or neutral) polymeric molecules have been suggested for the use as biomaterials. For more thorough review please refer to [55]. The architecture of the molecules giving rise to the hydrogel network can be varied regarding functionality and distance between the functionalities. Whereas the increase of functionality gives rise to higher cross-link density at constant polymer concentration, the increase in distance between functionalities will result in larger pore size. The polymer chemical composition largely influences the behavior of the material in aqueous environments. Whereas polymers containing ionizable pendant groups such as Copolymer networks of poly(methacrylic acid) grafted with poly(ethylene glycol) P(MAA-g-EG) respond with shrinkage or swelling, uncharged polymers are indifferent towards changes in buffer pH [123]. The use of block-copolymers consisting of hydrophilic and hydrophobic domains, such as poly(ethylene glycol)-bl-poly(propylene glycol)-bl-poly(ethylene glycol) (PEG-PPG-PEG), result in a hydrogel system, which due to a shift in temperature, is swelling or shrinking [124]. The copolymerization of materials building blocks which are temperature or pH sensitive results in hydrogels that responded to both stimuli [125].

Crosslinking mechanisms

For in vitro and in vivo applications, cross-linking of hydrogels must be performed under conditions which are not affecting cell viability. Hydrogels can be created by establishing affinity interactions or by chemical polymerization and depending on the affinity of physical interactions the stability of hydrogels can be modulated. Relatively weak interactions can be employed to form hydrogel systems which are responsive to stimuli such as glucose or antibiotics. Additionally they can result in hydrogels which are self-healing. The stability of chemically cross-linked hydrogels can be modulated by the introduction of linkages which are sensitive or insensitive towards hydrolytic degradation, proteolytic digestion, or reducing conditions. Chemical cross-linking today is mostly done by radical polymerization using photoinitiators, step-growth polymerization (Michael-type reaction) or enzymatic reactions presenting a spectrum of possibilities to choose for specific applications. Problems associated with cross-linking are lack of substrate specificity, cytotoxicity and reaction time. Although some photoinitiators have been associated with cytotoxicity, for a number of them the concentrations used are in a range where they are not compromising cell viability [126, 127]. Michael type reactions have been shown to have high substrate specificity and fast reaction kinetics under physiological buffer conditions. Recently, the even more selective native chemical ligation which involves a thioesther and an N-terminal cysteine has been used for the formation of hydrogels [128]. Also copper free click-reactions are now being developed, which have a good substrate specificity and improved

reaction kinetics [129]. Alternatively, enzymatic reactions which are known to have a very high substrate specificity have been used to form hydrogels [128] being highly compatible with the preservation of active growth factors.

The mechanical properties of hydrogels rely on hydrogel architecture, cross-linking efficiency, swelling behavior, initial concentration of monomers, and stoichiometry of reactants. Generally, higher polymer concentrations with high branching and short arm length can potentially lead to highly cross-linked hydrogels. Such hydrogels, with increasing hydrophilicity of the backbone polymer, can take up large amounts of water and thus will be mechanically strong. Factors that can weaken the mechanical properties of the hydrogel are for example limited cross-linking efficiency for example due to unbalanced stoichiometry of reacting groups, competing reactions, or suboptimal reaction conditions (pH, salt concentration). For more thorough review please refer to [56].

Synthetic hydrogels with engineered biological functions (static/controlled by cells)

As mentioned above, synthetic hydrogels are devoid of biological functions and thus provide a blank canvas for the engineering of materials with tightly controlled properties. Early experiments provided evidence that cells 3D encapsulated in such small porous (pore size is typically in the nm range) hydrogels cannot spread migrate, proliferate, and survive long term [130]. It has been demonstrated that the modification of the hydrogel backbone with biological building blocks such as cell adhesion sites and the introduction proteolysis sensitive backbone elements is necessary for the 3D culture of cells. These minimal modifications allow the encapsulated cells to locally digest the hydrogels, form small pores and move via complex processes via substrate adhesion and retraction of cell extensions. Of course, cells from different lineages have different requirements regarding the presented biological functional building blocks, which is where the challenges arise but also where novel hydrogel platforms with tunable properties prove their worth.

Modularly designed platforms as artificial extracellular matrices

Although synthetic hydrogels make it possible to study cellular response to isolated biological parameters, up to date only few hydrogels developed for 3D cell culture allow the independent tuning of properties such as biochemical signals and mechanical stiffness. Such materials have been created for example based on click chemistry [131], peptide self-assembly [132] and interpenetrating polymer networks [133].

In order to create a modular artificial extracellular matrix platform where matrix properties can be modified almost independent of each other, the factor XIIIa-catalyzed crosslinking scheme of fibrin clot involving the formation of a covalent isopeptide bridge between Gln and Lys residues by the enzymatic action of the transglutaminase factor XIIIa was employed [26]. Star shaped PEG-vinylsulfone molecules were functionalized with two peptides acting as substrates for FXIIIa via a Michael type

addition reaction, thus creating a homogenous synthetic hydrogel with fibrin-like biomolecular characteristics upon crosslinking with the enzyme. Simultaneously with the hydrogel crosslinking, growth factors, adhesion peptides or other biological entities functionalized with either of the FXIIIa substrates can be incorporated in a controlled manner (Figure 1.3A). By engineering of the PEG backbone to contain matrix metalloproteinase (MMP) or plasmin sensitive sites, the biomimicry of this system can be further extended (Figure 1.3B). This platform (called TG-PEG from here on) has so far been used to elucidate the effect of physical and biochemical matrix properties on processes such as cell migration, proliferation, spreading and angiogenesis and for creating of structured microenvironments as discussed later.

Usefulness of modularity can be elucidated for example by the case of cell migration in 3D which has so far mostly been investigated in naturally occurring materials [134]. Findings from such experiments are limited in terms of studying the effect of biochemical and biosphysical parameters since they cannot be decoupled from each other. For example, the effect of the ECM on the choice between proteolytic and nonproteolytic cell migration remains to be exhaustively answered [63]. Some light could be shed on the topic by using the TG-PEG hydrogels [135], which are devoid of microstructure therefore essentially nonporous for cells, as cross-linked polymers create pores in the range of tens of nanometer. By harnessing the modularity of this system in terms of protease sensitivity and stiffness, it was found that migration behavior was strongly dependent on matrix stiffness, with two regimes identified: a nonproteolytic migration mode dominating at relatively low matrix stiffness and proteolytic migration at higher stiffness. In non-degradable matrices with low stiffness, single cells could overcome the resistance of the matrix by engaging in a degradation-independent three-dimensional migration mode.

Similarly the TG-PEG system could be used for studying cell proliferation in 3D in response to selectively altered matrix characteristics [136]. To illustrate the difference in cell behavior in naturally occurring materials and fully synthetic hydrogels, fibroblasts were studied in collagen and TG-PEG hydrogels. The main physical difference between the two systems was that the PEG gels are purely elastic, whereas collagen gels generally display viscoelastic behavior due to the physical entanglement of normally freely moving fibers. Cells are able to dislocate collagen fibers leading to predominantly physical modification of the matrix. The PEG gels on the other had are mainly modified by biochemical processes, making it possible to systematically study such phenomena and their implications to other cellular processes, such as proliferation. By exploiting the modularity of the TG-PEG system, this study revealed that in spite of matrix sensitivity to proteases and the presence of cell-integrin binding sites, proliferation in 3D was hindered by high stiffness (elastic modulus >1200 Pa).

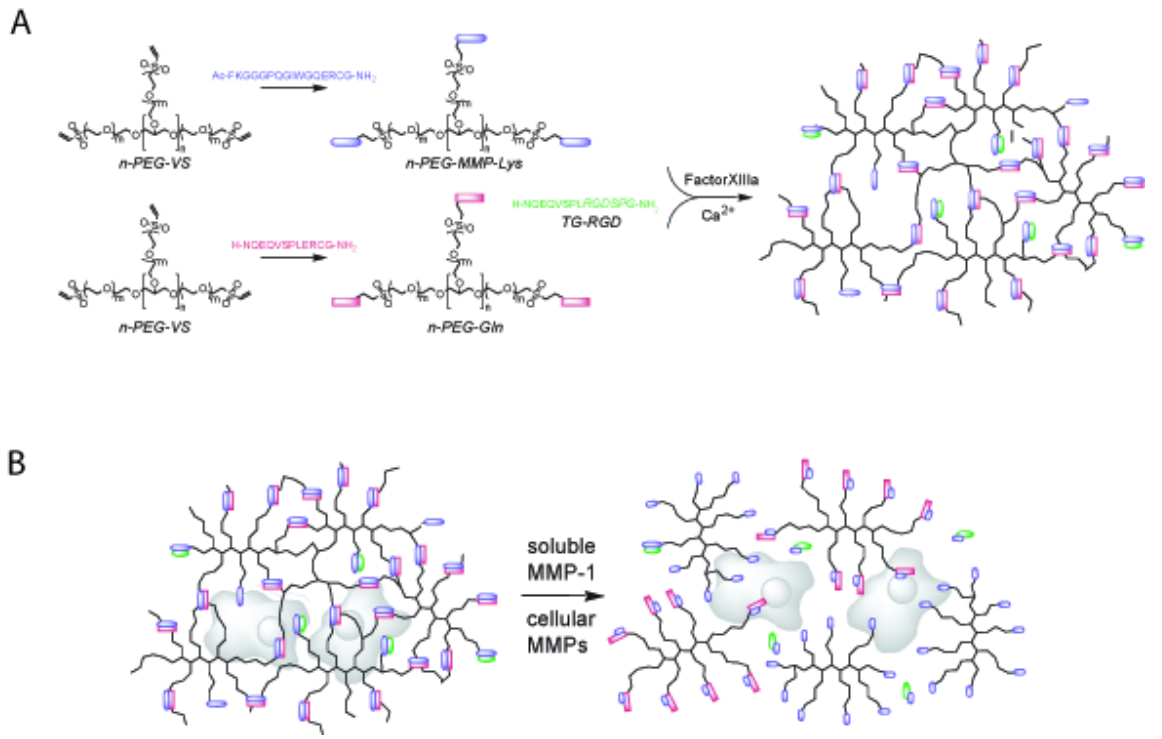

Figure 1.3 Factor XIIIa-catalyzed PEG hydrogel formation and degradation. A) The transglutaminase enzyme factor XIIIa was used to cross-link two multiarm PEG–peptide conjugates, n-PEG-MMP-Lys and n-PEG-Gln (here, n = 8), in combination with a cell adhesion peptide, TG-Gln-RGD, to form multifunctional synthetic hydrogels. B) Gel degradation by cell-mediated proteolysis (reprinted with permission from [26]).

Adhesion and degradation sites

Naturally occurring ECM contains numerous proteins and glycans which, dependent on the status of a tissue, are differently composed and thus provide different sets of adhesion domains. Ruoslahti et al. have found that a triple amino-acid domain derived from fibronectin is sufficient for the integrin mediated adhesion of cells [137]. Since then many cell-adhesive peptides, mostly derived from collagen, fibronectin, or laminin, to be linked into hydrogels have been described and used for the engineering of cell-instructive biomaterials (reviewed in [138]).

The polymeric backbone must provide space to cells in order to proliferate, deposit cells own matrix and move. Degradability of the material can be achieved through spontaneous dissolution of the polymer as for example by the incorporation of poly(lactic acid) (PLA) within the polymer structure or the use of acryl-sulfhydryl bonds [139]. These modes of degradation are materials autonomous properties. Materials stability is critical to appropriate tissue formation, as too fast degradation results in loss of structural and mechanical support, and too slow degradation will inhibit cell for example cell ingrowth and function. To render materials with tunable, cell-responsive degradation properties, polypeptides derived from occurring naturally protease sensitive sequences have been adapted and

incorporated into linker molecules of the backbone [130]. Recently, a number of peptide sequences with are recognized and degraded with different efficiencies (kcat) have been described [140, 141]. The use of substrate with very well defined degradation properties clearly will allow the fine tuning of materials properties towards specific applications.

Incorporation and cell mediated release of growth factors

Tissue development and regeneration depend on tightly coordinated spatial and temporal growth factor signals and recombinant growth factors have been widely proposed for therapeutic use in the regeneration and repair of diseased tissues. Increased knowledge on growth factor signaling as well as advances in recombinant protein engineering and production, have opened new possibilities is constructing artificial extracellular matrices. Strategies employed to immobilize growth factors in synthetic hydrogels are summarized in Table 1.1.

When growth factors are purely physically entrapped in the hydrogel matrix, availability for cells in space and time is determined by passive diffusion and coupled to hydrogel degradation. If not replenished in the culture medium, activity decreases over time. Diffusion can be controlled via modifications either on the hydrogel network properties or to the growth factor by which the affinity to the matrix, bioactivity, stability and bioavailability can be modified [142].

Effect of soluble factors on cells cultured in 3D can be studies in any hydrogel material, either by encapsulation to the material along with the cells or by addition to the growth medium, but for more sophisticated biomimicry, systems enabling the tethering and controlled release of growth factors are needed. Immobilization strategies allow the construction of gradients or localized areas where the factor is present, making it possible to more accurately recapitulate physiological situations. Natural ECM acts as a reservoir for growth factors from which they are released by cellular remodeling, which is often used as a release strategy in synthetic systems in addition to temporally controllable triggered release (Figure 1.2A). In the following paragraphs, growth factor immobilization strategies for synthetic hydrogels are discussed first focusing on covalent tethering and then moving to affinity based systems (Figure 1.2B).

Covalent immobilization

Covalent tethering of growth factors to synthetic or biologically derived hydrogels has been achieved by either chemical modification or genetic engineering of the factors to contain functional groups such as thiols, acrylates, azides and Gln-tags (Table 1.2). Initial chemical conjugation approaches utilized for example homobifunctional PEG-based crosslinkers with terminal and primary amine selective succinimidyl groups [106, 143], which could serve both as hydrogel crosslinking entities and as means to incorporate growth factors. Another chemical conjugation strategy was based on hetero-bifunctional N-hydroxysuccinimide (NHS)-PEG-acrylate linker, which could be used by first modifying the factor of interest with the amine specific NHS group reaction and subsequently coupling the

acrylated biomolecules into PEG-diacrylate networks by photopolymerization [144, 145]. Also click chemistry has been successfully used for covalent immobilization of growth factors into synthetic hydrogels [146].

The downside of these broadly applicable strategies, such as the reaction of NHS with any accessible lysine or the N-terminus, is their lack of specificity. The exact site and number of modifications are difficult to control and may have drastic effects on the growth factors bioactivity [147]. More site specific strategies have been realized by engineering recombinant proteins with additional cysteines as the abundance of reduced cysteines is inherently low in proteins and such modifications render them more susceptible to, for example Michael type reaction with vinylsulfone groups of PEG macromers [148]. Another possibility for achieving high site specify while conserving bioactivity is employing enzymatic reactions as discussed next.

The TG-PEG system was developed having in mind the goal of a flexible and modularly designed artificial extracellular matrix allowing site specific and stable immobilization of growth factor proteins into the gel network, without compromising the protein's bioactivity. Most straightforward way of doing this is by producing growth factors bearing a substrate for FXIIIa, as has been shown for VEGF [149]. VEGF121 was engineered to contain the glutamine acceptor substrate NQEQVSPL, derived from the N-terminus of α2-plasmin inhibitor (α2PI1-8) (termed Gln). This Gln-VEGF121 could be covalently incorporated into the hydrogel and released in controlled manner by the cleavage of the MMP sites in the PEG backbone during cellular matrix remodeling. The ability of the matrix bound Gln- VEGF121 to stimulate angiogenesis was evaluated in the embryonic chick chorioallontoic membrane (CAM) assay and the response to it was found comparable to that of native, diffusible VEGF121, indicating that this sequence-specific mode of morphogen affixation in aECM does not compromise a morphogen's activity, at least not in the case of VEGF. In contrast to conventional chemical bioconjugation schemes, the site-specificity of the enzymatic reaction gives precise control over the conformation of the immobilized molecules and results in no significant loss of its bioactivity. This may be crucial for applications involving sensitive transmembrane proteins (e.g. Notch ligands or Cadherins) in order to recapitulate cell–cell interactions via aECMs.

Affinity immobilization

In addition to covalent immobilization strategies, affinity interactions are another possibility for growth factor incorporation. These strategies take advantage of the interactions naturally occurring between growth factors and ECM components, such as heparin, chondroitin sulfate, hyaluronic acid and fibronectin. Thus they do not require direct chemical or genetic modification of the protein offering high degree of flexibility. Synthetic hydrogels have been modified with heparin via for example EDC/sulfo-NHS chemistry [150] or with streptavidin to allow the immobilization of biotinylated factors [151]. Also prefabricated affinity linkers, allowing simultaneous addition of growth factors and

cells during crosslinking [152, 153], as well supramolecular peptide gels with affinity sites for have been engineered [154].

Recently a very interesting approach based on the functional domains of fibrinogen was presented [155]. Recombinantly produced heparin-binding domain of fibrin(ogen) located at the N terminus of the fibrin(ogen) β chain (Fg β) was covalently linked into TG-PEG hydrogels and then used for the immobilization of various growth factors, for example FGF-2 and PIGF-2. Overall, interaction of the linker with 15 different growth factors were established which all displayed K_D values in the nM range. The fibrinogen fragment functionalized TG-PEG gels were successfully used to deliver growth factors into full thickness skin wounds in mice which lead to faster wound closure and increased development of granulation tissue.

Table 1.2: Strategies for immobilization and release of growth factors in synthetic hydrogels (adapted from [83]).

Matrix backbone	Linker	Ligand			Ref.		
		Native form	Modification	Immobilization	Release from Matrix		
Linker							
PEG-DA	NHS-PEG-Acryloyl [1, 2]	TGF-ß1, bFGF, EGF	chemical	covalent	enz.	-	[144, 145, 156, 157]
PEG-DA	SMC-PEG-Acryloyl [1, 2]	PDGF-BB, FGF-2	chemical	covalent	enz.	-	[158]
PLEOF	PEG-azide [1, 2]	BMP peptide	chemical	covalent	enz.	-	[146]
MAC	EDC/sulfo-NHS [1, 2]	IFN-γ	chemical	covalent	enz.	-	[159]
PEG-VS	-	$VEGF_{165}$/ $VEGF_{121}$	Ligand-Cys fusion [1]	covalent	enz.	-	[148, 160]
PEG-TG	-	$VEGF_{121}$	Gln-Ligand fusion [1]	covalent	enz.	-	[149]
PEG-DA, (Hep/HA/Gtn)-SH	heparin	VEGF, bFGF, Ang-1, HGF,KGF, PDGF-BB	-	affinity	enz. / hydr. dissoc.		[76, 161-163]
PEG-DA, (Hep/CS)-SH	heparin	bFGF	-	affinity	enz. / hydr. dissoc.		[164, 165]
PEG-SBA, Hep-ADH	heparin	VEGF		affinity	enz. / hydr. dissoc.		[166]
PEG-NH2	heparin, EDC/sulfo-NHS	FGF-2	-	affinity	enz.	dissoc.	[150]
PEG-petide, heparin	-	VEGF	-	affinity	enz.	dissoc.	[167]
PEG-LMWH, PEG-$PF4_{ZIP}$	heparin	bFGF	-	affinity	enz.	dissoc.	[168]
PEG-LMWH, PEG-HIP	heparin	bFGF	-	affinity	enz.	dissoc.	[169]
PEG-SH, HMWH	heparin	bFGF	-	affinity	enz.	dissoc.	[170]
PEG-LMWH, VEGF	heparin	VEGF	-	affinity	enz.	dissoc.	[171]
PEG-DM, heparin-MA	heparin	bFGF	-	affinity	enz.	dissoc.	[172]
PEG-VS, Cys_3-peptide	heparin	BMP-2	-	affinity	enz.	dissoc.	[173]
PEG-DA	poly(AAC)-Cys-bFGF-bp[1]	bFGF	Cys-bFGF-bp	affinity	enz. / hydr. dissoc.		[174]
PEG-TG	Gln-ZZ[1]	IL-4	Ligand-Fc fusion	affinity	enz.	-	[152]
PEG-TG	bead-Novo. GyrB-ZZ	PDGF-BB	Ligand-Fc fusion	affinity	enz.	Novo.	[175]
PEG-TG	Gln-GyrB[1]/Novo./GyrB-ZZ	FGF-7	Ligand-Fc fusion	affinity	enz.	Novo.	[176]
PEG-TG (Caged-Lys)		PDGF-BB, $VEGF_{121}$	Ligand-Fc fusion	light/covalent	enz.		[177]
PEG-TG		$VEGF_{121}$	Ligand-GyrB fusion	-	*		[177]
PEG-TG	Fg□□□□□□□□□	FGF-2, PlGF-2	-	affinity	enz.	dissoc.	[155]

[1] reacting with matrix; [2] reacting with Ligand; * Coumermycin induced ligand dimerization;

Abbreviations: enz., enzymatic; hydr., hydrolyzed; dissoc., dissociation; Novo., novobiocin; Gln, NQEQVSPL-peptide; NHS, N-hydroxysuccinimide; EDC, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide;poly(AAC)-Cys-bFGF-bp, poly(acrylic acid) modified FGF-2 binding peptide; PEG-NH_2, PEG-amine; PEG-SH, PEG-thiol; Hep, Heparin, HA, Hyaluronan; CS, Chondroitinsulfate; GEL, gelatine; PEG-SBA, N-hydroxysuccinimidyl ester PEG-bis-butanoic acid; $PF4_{ZIP}$, heparin binding, coiled-coil peptide; HIP, peptide sequence from heparin interacting protein; DM, Dimethacrylate; Cys, cysteine; VS, vinyl sulfone; PEG-TG, transglutaminase FXIII formed PEG hydrogel, Fg□□□□□□□□□□ N-terminus of the Fibrin(ogen) □□chain; MAC, methacrylamide chitosane; Coum., Coumermycin.

In order to develop a flexible affinity based strategy for the conjugation of growth factors to the previously described synthetic TG-PEG platform, a linker peptide consisting of the TG-domain and two repeats of the synthetic protein A analog (Z) was designed [152]. The TG-domain of this TG-ZZ peptide can be covalently bound to the Lys-PEG component of the TG-PEG system by the factor XIII mediated reaction. Due to the high affinity of the ZZ peptide ($4.8 \times 10^{-8}\ M^{-1}$) to the fragment crystallizable (Fc) region of antibodies, Fc-tagged growth factors can be immobilized within the hydrogel network. Taking advantage of the high affinity of this interaction, the linker can be used to directly capture Fc-tagged proteins produced by mammalian cells encapsulated in the hydrogels. This creates a versatile platform to concentrate and purify large panels of recombinantly expressed growth factors for cell-based assays to identify novel regulators of cell behavior

The fusion peptide Gln-ZZ was expressed in bacterial cultures and its functionality demonstrated by capturing FITC-labeled IgG antibody from a solution into a linker containing hydrogel and retaining it throughout extensive washing. With the ultimate goal of growth factor immobilization in mind, the functionality of growth factor or cytokine-tethered hydrogels as cell-instructive matrices was demonstrated using the anti-inflammatory cytokine Interleukin-4 (IL4). Protein-capture hydrogels and control hydrogels were patterned and placed in cultures of human embryonic kidney (HEK)-293T cells expressing IL4-Fc and after washing, the activity of the captured IL-4 was demonstrated by a reporter cell line. ZZ-linker modified hydrogels could be used for providing a reservoir of biomolecules, which can actively stimulate cellular responses, either in their matrix-immobilized form or after cell-mediated proteolytic degradation of the hydrogel in a soluble form.

Local 3D structuring of hydrogels

Homogenous biomimetic hydrogels can only be used to address biological questions in a simplified manner, which for certain situations, such as high throughput screening platforms, can be sufficient or even desired. By creating spatially defined heterogeneous microenvironments, more sophisticated models recapitulating higher levels of biological complexity can be realized, helping to bridge the gap between current in vitro and vivo models. Bulk functionalization of synthetic hydrogels has been widely described in literature, but this alone is not sufficient for mimicking the natural ECM. Thus,

efforts have been made for evolving hydrogels for cell encapsulation from physically and biochemically homogenous materials into highly spatially defined environments structured in multiple size scales.

Heterogeneity can be introduced in 3D in terms of local biochemical composition (e. g. adhesion ligands and growth factors) or by introducing structural patterns (e. g. variations hydrogel composition, empty, or cell filled spaces). Hydrogels with such features have been produced using techniques such as self-assembly of prefabricated building blocks (e.g. microscale hydrogels or fibers) [178-182], casting [183], additive manufacturing (e.g., printing and layer-by-layer deposition) [184, 185], photo-patterning [186, 187], and microfluidics [188, 189]. Problems with spatial patterning of hydrogels in 3D arise from their thickness and extensive swelling.

Next, two approaches, namely the combination of printing with layer-by-layer deposition for modelling vascularized bone, and electrochemical control of polymerization for creating channel structures and gradients of biomolecules, are discussed in the context of the TG-PEG system.

Generation of functional vascularized tissues remains the holy grail of tissue engineering, and in order to address related questions using in vitro models, structuring ECM mimicking materials and cells comes into play. In the context of bone, combining the TG-PEG hydrogel system as an artificial extracellular matrix with matrix structuring technologies, namely robotic printing and layer-by-layer deposition, the role of biological constituents in the vascular bone microenvironment could be systematically evaluated [184].

Feasibility of layer-by-layer deposition of TG-PEG was demonstrated by combining 200 μm cell containing hydrogel blocks with confluent layers of cells (Figure 1.4A). Since gel layers can be formed in the presence of cells or used as surfaces for the seeding of cells, even such a simple layering approach allows for a large variety of cell and matrix combinations. For introducing more heterogeneity with each gel layer, robotic printing platforms using a contact-spot arraying technology with a lateral precision of approximately 5 μm and robotic pipetting with a lateral precision of approximately 100 μm were used. With these systems spatially segregated hydrogel drops of ca. 100 μm of diameter and 20 μm height could be formed (Figure 1.4A). The size of the droplets can be modified by adjusting different parameters such as the pin-head size, the amount of dispensed liquid or using repetitive printing steps.

Initially, conditions supporting the viability and spreading of osteoblasts (MC3T3-E1) and osteocytes (MLO) or blocking their migration were defined in terms of hydrogel stiffness and presentation of RGD ligand. The effect VEGF and mono-, co- and tri-cultures on the formation of capillary like network in 3D was then assayed. Using a fully synthetic hydrogel platform, it was possible to provide evidence on the importance of single components on angiogenesis. It was demonstrated that besides the adhesion ligand RGD and the pro-angiogenic growth factor VEGF, the presence of both stromal cells appears to be critical for endothelial cells (ECs) to efficiently form tube-like structures in artificial environments.

Once the culture conditions were defined, the three cell types were assembled together to create spatially organized vascularized bone tissue-like constructs. A supportive layer of 4% PEG hydrogel covered with a monolayer of MC3T3-E1 was used as a basis for the construct and on top of this, human umbilical vein endothelial cells (HUVECs) were subsequently positioned in 1µl-sized hydrolytically and proteolytically degradable PEG-acrylate gels. As PEG-acrylate degrades rapidly the drops were formed without RGD ligands to prevent high concentrations of soluble peptides which could potentially interfere with HUVEC viability. As a final step, a covering layer of PEG containing human osteocytes was added and the constructs were allowed to remodel for 10 days under the previously optimized conditions. Analysis of the constructs revealed a dense and interconnected network of HUVECs and preosteoblastic cells surrounding the endothelial structures.

These findings demonstrate that simultaneous patterning of matrix components, ligands, and cells to direct cellular spreading and to form artificial tissue-like constructs allows for the dissection of biological questions related to tissue remodeling and morphogenesis, and their independent study in a fully controllable artificial environment.

In addition to the strategies described for biological functionalization of TG-PEG hydrogels, electrochemical polymerization has been employed for eliciting spatial control over the crosslinking in 3D [185]. This is based on the FXIII-catalyzed crosslinking reaction being pH-dependent and occurring with highest efficiency at pH 8, while being damped at more acidic or basic conditions [149]. The proteolysis of water induced by electric current was used to locally decrease (at the anode–electrolyte interface) or increase pH (at the cathode–electrolyte interface) and thereby prevent or promote hydrogel formation. This method was successfully used for creating microchannels within TG-PEG hydrogels in a PDMS mold using an anodically polarized tungsten wire as a template. By locally controlling the polymerization around the wire, it could subsequently be removed with disrupting the gel, which was the case without the current due to the strong adhesion of the PEG to the metal surface.

Electrochemical control of gel polymerization could also be used to form complex, locally functionalized 3D microenvironments in TG-PEG by a sequential approach (Figure 1.4B). This was demonstrated by first polymerizing FITC functionalized gel around a cathode wire, followed by thorough washing to remove the unreacted components, and finally backfilling the mold with a TRITC functionalized gel while creating a second channel. The area between the two channels was thus functionalized with both molecules and the sides with one molecule alone. When injected into the preformed channels, various cell types such as bone-marrow-derived mesenchymal stem cells (MSCs), preosteoblasts and fibroblasts were able to invade into the surrounding hydrogel mass, which will enable the study of cellular responses to different (graded) microenvironments. HEK cells expressing a yellow fluorescent protein (YFP) upon being exposed to IL-4 were used for demonstrating such spatial specificity of the channel surrounding microenvironments after functionalization with hydrogel bound IL-4. In conclusion, by electrochemically controlling the enzymatic cross-linking of TG-PEG hydrogels it

was possible to create spatially defined 3D microenvironments containing various tethered biomolecules and thereby control local cell functions.

Further understanding on how to best structure artificial microenvironments and automation of these procedures could lead to large scale production of reproducible tissue-like constructs suitable for high throughput screening approaching in vivo complexity, with yet a simple readout for evaluation.

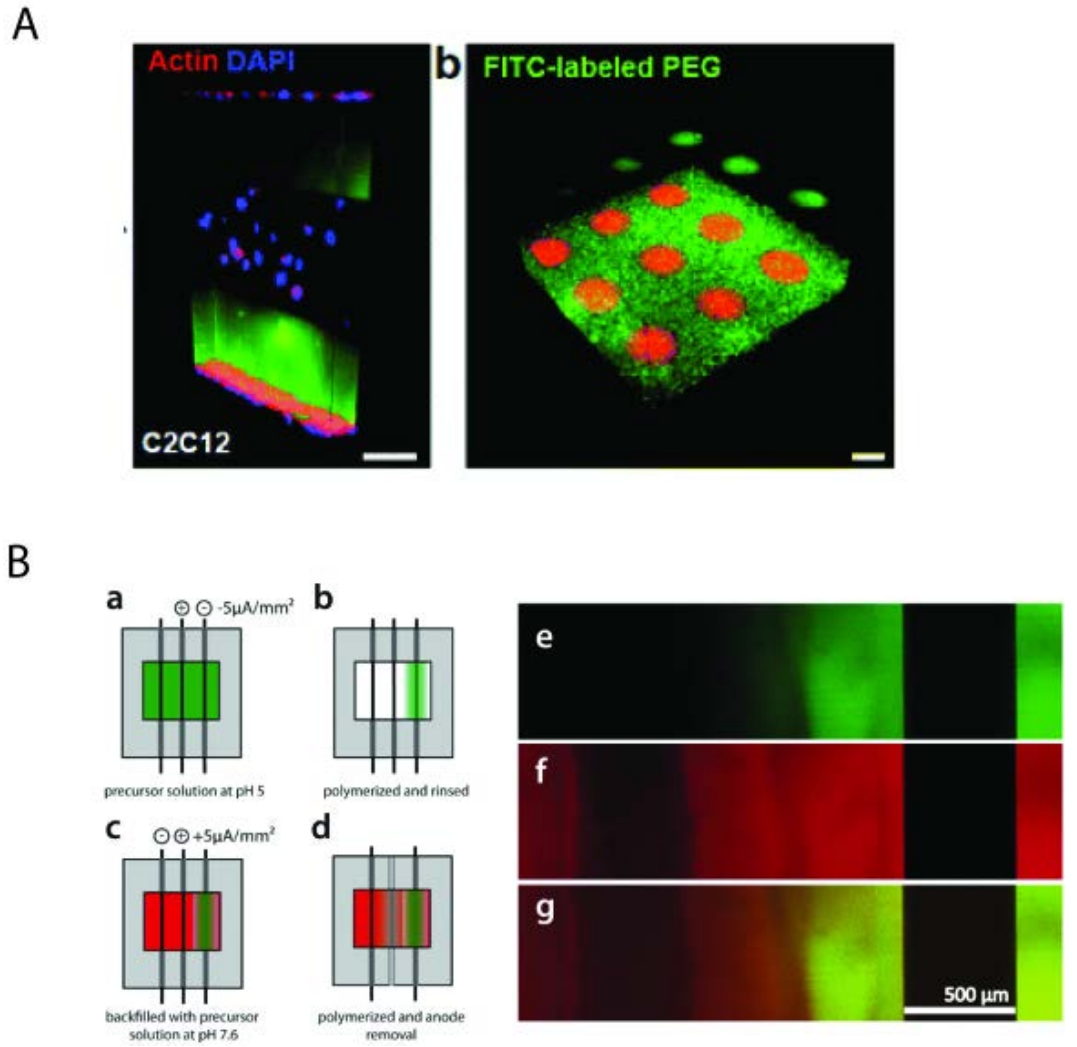

Figure 1.4 Structured microenvironments in TG-PEG hydrogels. A) Homogeneous layers of C2C12 cells and cell-containing or FITC-labeled gels (gel mass either in green or unstained) and different fluorescently labeled hydrogels arranged by robotic printing in defined positions on top of different layers of hydrogel (scale bar 100 µm). B) Schemes depicting the production of an engineered microenvironment containing two different fluorophores using electrochemical structuring, and confocal microscopic images of the resulting microenvironment. Reprinted with permission from [184, 185].

Spatio-temporally controllable dynamic microenvironments (external control)

Native ECMs undergo tightly controlled, constant remodeling resulting in liberation of growth factors or other bioactive entities as well as changes in matrix mechanical properties. Although the above presented synthetic hydrogel matrices have largely overcome the limitations of naturally occurring materials by handing over the control of materials physical and biochemical properties to the scientists, they still do not allow modulating the availability of extrinsic signaling molecules and material mechanics in time. Possible trigger mechanisms include changes in temperature [190-192], pH [48, 193], electromagnetic stimulation [194] or taking advantage of bioactive compounds [122, 195-197]. Most feasible in vitro approaches reported so far are based on light or using diffusible molecules interfering with interactions within the hydrogel, so the focus in this chapter is on such approaches. Light is an attractive candidate for spatio-temporal manipulation of hydrogels because its temporal patterning resolution can be controlled at single cell or even subcellular scale and in the visible and long wave UV region has little effect on cell viability and behavior.

Dynamic presentation of biological cues

Bioactive molecules such as growth factors can have cross-reactive effects on different cell types, making timing of their presentation in vivo as important as spatial control. This has promoted the development of experimenter-controlled culture matrices, in which adhesion sites and growth factors can be patterned and released on demand to change the biochemistry of the microenvironment to mimic processes such as differentiation.

Light-sensitive molecular building blocks can be attached to hydrogel networks to generate artificial ECMs in which the properties of microenvironments could be specifically modulated by light exposure [198]. Previously only small synthetic peptides have been the target of photopatterning approaches because the employed chemical crosslinking or bioconjugation reactions, using nonspecific chemistries, often damage full length proteins. Photochemical caging strategies have emerged as a means to use light for controlling the temporal presentation of larger biological compounds without impairing their bioactivity.

Reversible presentation of biological entities has been reported based on the combination of two orthogonal, biocompatible wavelength specific photoreactions, namely thiol-ene reaction for photocoupling and photoscission of an *o*-nitrobenzyl ether for later release from PEG hydrogels [199]. Spatial control of both reactions by controlling the introduction and removal of functional groups, was achieved at a subcellular scale in 3D by focusing pulsed laser light within the hydrogel volume with resolution of 1 μm in the *x*-*y* plane and 3–5 μm in the *z*-plane. Mouse embryonic fibroblast (NIH 3T3) cells were seeded on gels with photopatterned adhesive regions from where they could subsequently be detached by the removal of the adhesive ligand after being first allowed adhere and spread for 24 hours.

The effect of temporal ligand presentation on stem cell phenotype is an important question that could be studied by incorporation photocleavable adhesion molecules. A nitrobenzyl ether-derived moiety was acrylated by a pendant hydroxyl group [photodegradable acrylate (PDA)] and was subsequently attached with a pendant carboxylic acid to poly(ethylene glycol) (PEG)–*bis*-amine to create a photocleavable cross-linking diacrylate macromer. The cross-linker was the copolymerized with PEG monoacrylate (PEGA) by redox-initiated free radical polymerization to create photodegradable hydrogels [200]. Matrix bound photolabile RGDS peptide was used for investigating the effect of persisting versus temporally modulated RGD environment on hMSC viability and chondrogenic differentiation. Mimicking native chondrogenic differention, during which fibronectin is initially produced but subsequently down-regulated and replaced by glycosaminoglycans (GAGs) and type II collagen (COLII), was achieved by removing RGD sites after initial culture.

By utilizing multiple modes of crosslinking of acrylated hyaluronic acid in a sequential manner, degradability of 3D matrices could be regulated temporally [201]. During the primary polymerization step hydrogels with adhesive sites and MMP-cleavable dithiol crosslinkers were formed via an addition mechanism, which still left a portion of the acrylate groups unconsumed. At this stage the matrices were permissive to remodeling and migration but by localized light exposure (using a photomask or a focussed laser) could be further cross-linked rendering them unfavorable for such cellular functions. Robust spreading of mesenchymal stem cell was observed within the permissive areas and found to be dependent of the relative amount of each crosslinking mode whereas in highly cross-linked "inhibitory" hydrogels cells remained rounded. Such differences in cellular morphology could be useful for studying signaling mechanisms during spatially controlled differentiation of encapsulated stem cells.

Spatiotemporal regulation of biological compounds in synthetic microenvironments, trapping them in cages and subsequently uncaging them the site of interest, has been shown to allow for a nearly instantaneous manipulation of the bioactive compound concentration [202]. Small signaling molecules and chemically synthesizable peptides have been successfully caged based on chemical modification of the molecules using a photo-removable protective group. This strategy is in most cases not compatible with large proteins, such as growth factors, which require a tailored caging procedure. Also caging is often complicated due to difficulties in achieving site-specific chemical modification.

FXIII crosslinkable TG-PEG hydrogels could be rendered photosensitive by masking the active site of one of the FXIIIa substrates with a photolabile cage group [177], namely nitroveratryloxycarbonyl (Nvoc), which by its broad absorption in ultraviolet/visible around 350 nm is advantageous in terms of penetration depth and minimal DNA and biomolecule damage [202]. Caged Lys-substrates within the polymerized hydrogel network could be subsequently released enabling highly localized enzymatic biomolecule tethering. This system was exploited for light activated enzymatic gel patterning to manipulate the behaviour of live cells within the hydrogel microenvironment. 3D invasion of human

MSCs was chosen as a physiologically relevant model, illustrating injury induced cell recruitment. RGD, the recombinant fibronectin fragment $FN_{9-10}$and platelet-derived growth factor B were engineered to contain the TG-peptide for crosslinking into the matrix. Microtissues of MSCs were encapsuled into MMP- and photosensitive TG-PEG hydrogels and a cuboidal pattern of uncaged TG-peptide was then created on one half of the microtissue. The enzymatically immobilized, fluorescently labelled RGD and FN_{9-10} permitted increased MSC migration within the patterned areas. This demonstrated that light-activated enzymatic gel patterning can be exploited for manipulating the behaviour of live cells in three dimensions directly in culture. Same strategy was employed on hyaluronic acid hydrogels [203].

To create a generic retain and release system applicable to any arbitrary protein with an fc-tag, a strategy based a pharmacological cage was employed [175]. The cage was formed by covalently coupling novobiocin to an epoxy-activated agarose matrix via a nucleophilic addition reaction. An adaptor protein consisting of the novobiocin-binding domain of the bacterial protein gyrase subunit B (GyrB) fused to the IgG-binding domain ZZ (derived from Staphylococcus aureus protein A) was used for anchoring proteins via an fc-tag. Protein contained in the cage could be subsequently released by addition of free novobiocin competitively inhibiting the binding between the adapter and the cage. Caging and rapid uncaging was demonstrated by regulating MSC migration out of migrotissues in 3D hydrogels by controlled release of caged Fc-PDGF.

Another pharmacochemical approach based on controlling the activity of a growth factor via dimerization was used as a basis for an inducible ON-OFF regulation for cysteine-knot growth factors such as VEGF [204]. The switch consisted of a chimeric protein with an engineered monomeric variant of the protein of interest fused to the inducible dimerization domain of the bacterial protein gyrase B (GyrB). The default state of the switch was OFF as the monomeric structure prohibits dimerization and thereby activation of the protein receptor. The switch could be turned ON upon the addition of coumermycin which binds to GyrB, leading to dimerization of the protein and thus the activation of the receptor and downstream signaling processes. The OFF state can be restated by the administration of novobiocin, upon which the single coumarine ring of the novobiocin competitively inhibits binding of coumermycin to GyrB, returning the protein to its monomeric state. ON-OFF regulation of the system was demonstrated by controlling the VEGF induced migration of HUVECs in PEG hydrogels in 3D. As coordination of endothelial cell migration is a key component in angiogenesis, methods to study its regulation are highly valuable for both basic research and tissue engineering.

Mechanically dynamic materials

Cells embedded in the extracellular matrix of tissues encounter and respond to ECM stiffnesses in the range of 0.2-1 kPa (brain) to 30-45 kPa (osteoid). They actively exert pushing and pulling forces on their surroundings, which results the activation of intracellular mechanotransduction pathways [205]. For example vascular endothelial cells experience different types of flow which directs

their behavior and stiffening of the ECM in liver drives liver fibrosis. Mechanics are also tightly coupled with stem cell fate demonstrated by the finding that mesenchymal stem cells are driven towards osteogenesis by a stiffer and towards adipogenesis by a softer hydrogel environment [206]. Despite the biological relevance of mechanically dynamic systems, there are only few examples described in the literature and almost none based on synthetic hydrogels. To circumvent cell induced changes in matrix properties over time, some work has been on culturing cells on them in 2D mechanically dynamic hydrogels by culturing cells on them in 2D to circumvent cell induced changes in matrix properties over time complicating the situation in 3D.

Hydrogel mechanics can be controlled for example by introducing chemical groups that can be cleaved by ultraviolet light leading to matrices that soften upon light exposure [200]. By copolymerizing a photocleavable diacrylate macromer cross-linker with PEG monoacrylate (PEGMA), using redox-initiated free radical polymerization, photodegradable hydrogels were created. The cross-link density of hydrogel networks could be reduced (by light) in presence of viable human mesenchymal stem cells allowing their transition from a round to elongated morphology. This same system could be used to direct cell migration by 3D patterning paths for encapsulated cells in real time.

In contrast to dynamically softening hydrogels, matrix stiffening, taking place for example during development and wound healing, might actually be more a biologically relevant phenomenon to mimic as it has been implicated regulate many cellular processes. A sequential crosslinking approach was used to create dynamically stiffening hyaluronic acid hydrogels [207]. Methacrylate functionalized hyaluronic acid was in the first step crosslinked via a Michael-type addition reaction using dithiothreitol (DTT). The remaining initial hydrogel could be further stiffened by radical polymerisation of the remaining methacrylate groups using a photoinitiator and ultraviolet light exposure. The system could be used to investigate cellular response to substrate stiffening in terms of cytoskeletal rearrangement and differentiation, though it was currently only reported on cells grown on top of the hydrogel instead of 3D.

The interaction of GyrB protein with novobiocin and coumermycin was utilized for synthesizing stimulus responsive polyethylene glycol (PEG) hydrogels [176]. Thiol containing GyrB was covalently grafted to multi-arm PEG-vinylsulfone molecules using a Michael-type addition reaction. Stable hydrogels could be formed by addition of the GyrB-dimerizing substance coumermycin. These hydrogels could again be dissolved in a dose-adjustable manner by the antibiotic novobiocin. This matrix could be used for cell growth either in vivo or in vitro, where the stimulus-responsive characteristics can be used to controlled release of growth factors or for dynamic tuning of the matrix mechanical properties.

With the incorporation of RGD motifs the hydrogel could support the adhesion and growth of human primary cells derived from gingival epithelial and connective tissue. No cytotoxic effects could be observed upon gel dissolution with novobiocin, which at 50μM concentration took place in 4 hours.

In order to add biological functionality with cell-instructive biomolecules like growth factors, the protein A-derived ZZ-domain was fused to GyrB (ZZ-GyrB), thereby allowing for the immobilization of proteins with an Fc-tag. Fibroblast growth factor 7 (FGF-7), a protein acting in a paracrine manner and being a key player in epithelial tissue regeneration was used as a model protein. Fc-tagged FGF-7 could be released in a dose and time dependable manner and it was also shown to retain its activity as demonstrated by the dose-related induction of proliferation of gingival mucosal keratinocyte.

Outlook and needs

In the previous sections we have described some of the currently available mostly homogenously engineered hydrogel systems to control cell function in 3D. However, morphogenetic processes (during tissue formation and regeneration) are highly orchestrated events, mastered by chemotaxis, differentiation, or proliferation of multiple cells from different lineages. These complex spatio-temporally regulated processes are driven by spatially restricted microenvironmental cues, consisting of growth factors, matrix components, and mechanical properties. In order to form 3D tissue mimetics and reproducible organoid culture systems such cues need at least to be partially recapitulated to initiate cell-autonomous tissue morphogenesis. Whereas in biologically derived, complex, and not easily amenable to engineering matrices, impressive tissue formation was observed, for fully defined hydrogels systems optimal matrix properties for many applications still remain elusive. Clearly, much has to be learned about the function of the native ECM and to integrate more specific integrin ligands into synthetic materials. There will also be a need for more and highly specific, modular building blocks for the selective incorporation of multiple different growth factors, crosslinking elements, degradation sites, and cell adhesion sites.

Nonetheless the many existing, sophisticated materials building blocks generated in recent years provide a growing toolbox for the creation of tailor made synthetic hydrogels and their integration with biological materials (Figure 1.5A). Such platforms are an exciting starting point for the assembly of complex 3D structured tissue constructs by the combination with emerging, highly sophisticated micro-manipulation techniques such as cell and materials printing devices, microfluidics, layer-by-layer assembly (Figure 1.5B), micromolding techniques, or preventive manufacturing. For manufacturing conditions using gel systems many critical issues will have to be solved, for example the evaporation of water and the consequent shift in hydrogel properties (due to change in polymer cross-link density due to increased concentrations) during printing. Also building blocks which are formed individually will likely suffer from different materials properties in the bulk and at the periphery. Such boundaries can largely influence the trafficking of cells and could restrict cell interactions and need to be carefully addressed.

Furthermore, in order to allow temporal, operator controlled, site and individual parameter specific manipulation of the system, different building blocks would ideally respond highly specific to triggers such as light with different wave length, shift in temperature or presence of minute quantities of small

chemicals (Figure 1.5C). Of course, improved precision of 3D culture systems must go along with the development of monitoring tools for cells and matrix components. Not only cell-based reporter systems, but also high-throughput automated image acquisition and image analysis algorithm systems as well as means to understand the remodelling of the provisional matrix components and the deposition of cell´s own ECM need to be followed.

In conclusion, we believe that "advanced" cell culture models hold great promise for the establishment physiological meaningful 3D tissue mimetics and the reproducible culture of organoids under highly defined conditions and amenable to investigator controlled manipulations. Such systems in future will not only be important intermediates between homogeneous 2D and 3D cultures and in vivo systems and thus could become tools applied to study basic biological questions as well as to validate lead compound during drug screening.

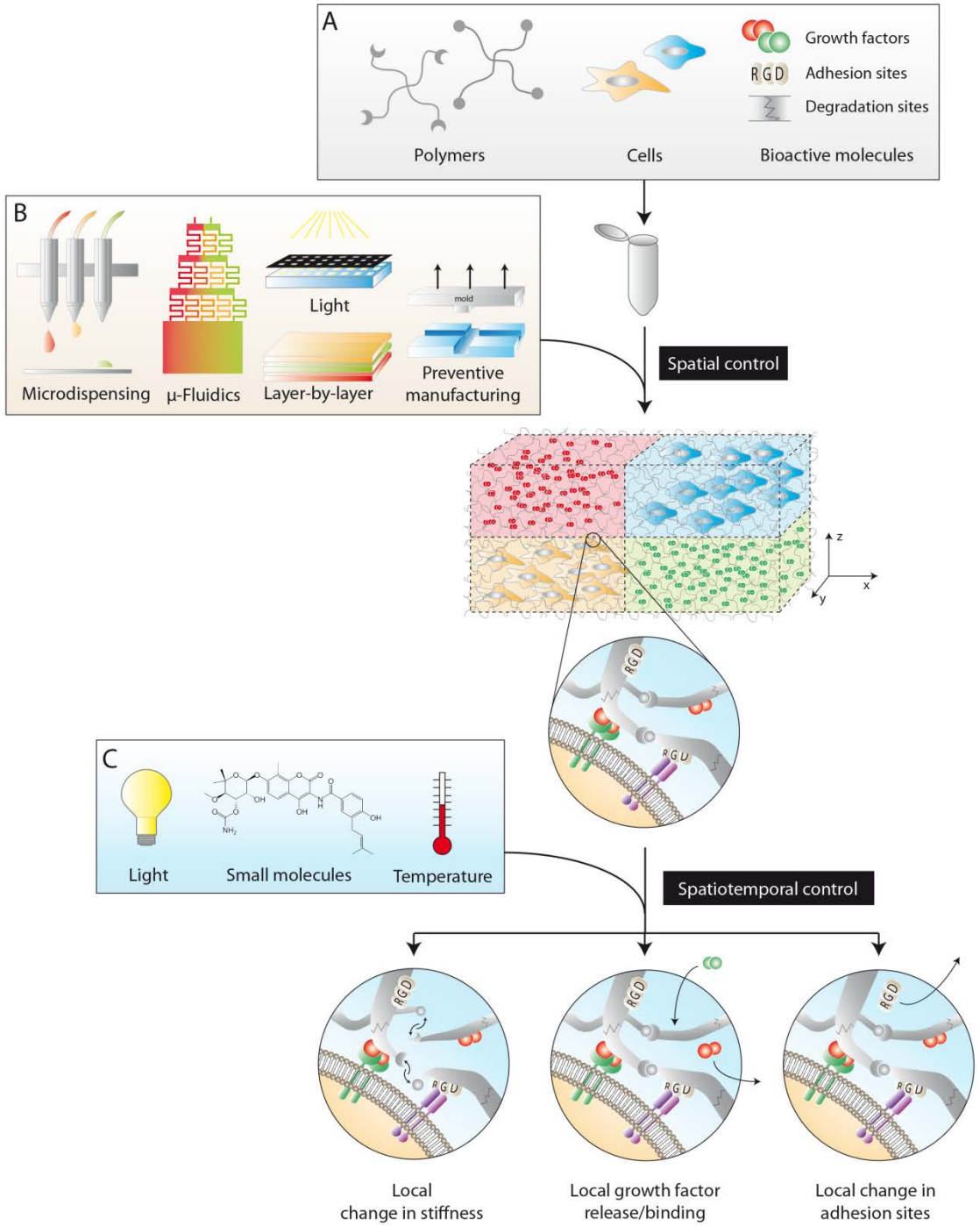

Figure 1.5 Strategy for creating 3D tissue models starting from A) combining biological elements with synthetic hydrogels, B) structuring these elements and finally C) eliciting spatiotemporal control over the system.

SPECIFIC AIMS

Aim 1

In vivo evaluation of the sealing capability and tissue interaction of mussel-mimetic tissue adhesive (mussel glue) in comparison to fibrin glue on punctured fetal membranes. Non-healing defects in the fetal membranes after fetoscopic procedures pose a considerable risk for fetal well-being. As a first step towards developing sealing or healing strategies for this clinical problem, decellularized human amnion membranes were tested as a plug together with a glue in a midgestational rabbit model. Performance of a biomimetic, synthetic PEG based glue functionalized with catechols (mussel glue) resulting in an excellent adhesion to wet surfaces was compared with commercially available fibrin glue. The aim was to evaluate the sealing properties of both glues and tissue resonse to them as well as whether the biological signals left in the decellularized scaffold could iniate healing of the punctured fetal membranes. This study is presented in detail in **chapter 2**.

Aim 2

In vitro screening of factors inducing recruitment, proliferation and ECM production of amnion membrane cells for fetal membrane healing materials. Results from **Aim 1** lead to an idea that a synthetic plugging material with specifically tailored biological and mechanical propertie might activate the cells in the amnion and induce a healing response. The goal was to use TG-PEG matrices in vitro together with soluble growth factors to identify signals which mobilize human amnion mesenchymal cells (hAMC) and also induce their proliferation and ECM synthesis in a 3D tissue mimicking environment. Identification of factors that support the formation of load bearing tissues by these cells would allow producing TG-PEG based growth factor presenting plugging materials for healing fetal membrane defects. Detailed experimental setup for screening of growth factors and the acquired knowledge are described in **chapter 3**.

Aim 3

Generation of autologous vascularized bone-like tissues using amniotic fluid cells as a single cell source. Isolation, culture and differentiation of amniotic fluid cells (AFC) has been reported in the literature. Aim 4 focuses on investigating whether these cells could used as a single cell source to produce autologous prevascularized bone constructs to treat conditions such as cleft palate. TG-PEG platform together with a specific mold design for producing channel structures within hydrogel matrices was used to create three dimensional prevascularized osteogenic constructs. The goal of was to first differentiate the AFC in the bulk hydrogel towards bone and produce a collagen rich mineralized matrix. Endothelial differentiated AFC were then seeded into the channel of this bone like

TG-PEG gel for vascularization. Fabrication, in vitro characterization and effect on bone healing in vivo of these constructs is reported in **chapter 4.**

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CHAPTER 2 Mussel mimetic tissue adhesive for fetal membrane repair: initial *in vivo* investigation in rabbits

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ABSTRACT

Iatrogenic preterm prelabour rupture of fetal membranes (iPPROM) remains the main complication after invasive interventions into the intrauterine cavity. The aim of this study was to evaluate the sealing capability and tissue interaction of mussel-mimetic tissue adhesive (mussel glue) in comparison to fibrin glue on punctured fetal membranes *in vivo*. A mid-gestational rabbit model was used for testing the materials. The fetal sacs of pregnant rabbits at day 23 were randomly assigned into experimental groups: Unoperated (negative control), unclosed puncture (positive control), commercially available fibrin glue with decellularized amnion scaffold (DAM), mussel mimetic sealant with DAM or mussel mimetic sealant alone. Evaluation was done at term (31 days gestation) assessing fetal survival, fetal membrane integrity and histology of the membranes. All plugging materials could be found at the end of the pregnancy and no adverse effects on the fetus or the pregnant does could be observed. Cellular infiltration could be seen in fibrin glue and DAM in contrast to mussel mimetic sealant which was only tightly adhering to the surrounding tissue. These cells were mostly of mesenchymal phenotype staining positive for vimentin. CD68 positive macrophages were found clustered around all the plugging materials, but their numbers were only significantly increased compared to negative controls for the mussel glue alone group. Mussel glue showed comparable *in vivo* performance to fibrin glue in sealing fetal membranes in the rabbit model. Taking into account its other favorable properties, it is a noteworthy candidate for a clinically applicable fetal membrane sealant.

Keywords: Fibrin sealant; fetal membrane repair; iatrogenic PPROM; rabbit model; mussel mimetic sealant

INTRODUCTION

Iatrogenic Preterm prelabour rupture of fetal membranes (iPPROM) remains a clinically unsolved problem after invasive prenatal procedures such as amniocentesis or fetal surgery. Randomized studies have shown the benefit of fetal surgery: fetoscopic procedures are the gold standard in treatment of twin-to-twin transfusion syndrome (TTTS)[1] and open fetal surgery for myelomenigocele (MMC) repair showed a better outcome than postnatal repair[2]. However iPPROM still remains coupled to these advances. Potential consequences include oligohydramnios-related pulmonary hypoplasia, chorioamnionitis and preterm delivery, posing a considerable threat to the well-being of affected fetuses[3]. Complications associated to it compromise the expected benefits of any intervention, making it a serious problem for modern fetal surgery.

Fetal membrane defects have shown no spontaneous healing[4] and though attempts to stimulate repair with naturally derived materials have shown some promise of success[5, 6], the *in vivo* instability of such materials limits their applicability. As fetal membranes only need to exist during pregnancy, bonding to wet surfaces and stability in physiological conditions seem to be more important properties for plugging materials than induction of healing and remodeling[7].

Previously, the synthesis of catechol functionalized poly(ethylene glycol) (cPEG) polymer, a mussel mimetic tissue adhesive (mussel glue) was described[8]. When employed in a murine model of pancreatic islet transplantation mussel glue's long term *in vivo* stability and good tissue integration was demonstrated by the absence of an inflammatory response. Consequently several injectable sealants, including mussel glue, were evaluated *in vitro* in terms of toxicity and bonding to fetal membranes with the goal of using the best candidate for closing iatrogenic membrane defects[9]. Mussel glue and commercially available fibrin glue (TissueCol Duo S, Baxter) performed the best and showed efficient, non-disruptive, non-toxic bonding to fetal membranes. Sealing capabilities and enzymatic degradability of both sealants have thereafter been further tested[9]. It was found that *ex vivo*-tested mussel glue sealed fetal membranes and resisted pressures reached during uterine contractions and that mussel glue was insusceptible to degradation by collagenase and plasmin as well as amniotic fluid.

Decellularized amnion membrane (DAM), previously was tested for sealing fetal membrane defects in rabbits[5]. Results from this study indicate that DAM application results in immediate plugging of the puncture site and mobilization of cells to the defect zone. The immobilization and stabilization of a scaffold with a gluing material stable under physiological conditions is thus hypothesized to combine sealing and healing effect and to provide the best outcome.

Based on these promising results with the mussel glue, the goal here was to take the next step in evaluating its suitability for the treatment of iPPROM using the mid-gestational rabbit[10] which has become the standard model for initial testing of fetal membrane sealing materials.

MATERIALS AND METHODS

All animal experiments were carried out in accordance with the current guidelines on animal welfare, and the experiments were approved by the Ethics Committee for Animal Experimentation of the Faculty of Medicine at KU Leuven, Belgium.

Mussel glue

The production and characterization of mussel glue, a cPEG lacking the primary amine of the DOPA amino acid, was performed as described elsewhere[8]. For the application of gluing material on punctured membranes, equal volumes of cPEG precursor solution (300 mg/mL in phosphate-buffered saline (PBS)) and sodium periodate solution (12 mg/mL in water) were mixed by pipetting. Sodium periodate initiated gelation, which was achieved when reactive dopamine groups formed covalent crosslinks[12].

Fibrin glue

Tissucol Duo S fibrin glue (Baxter) is widely clinically applied as haemostatic surgical sealant or an adjuvant to suture. It is a biologic two-component adhesive that forms by the mixing of human plasma cryoprecipitate solution with thrombin solution mimicking the last step of the natural blood clot formation[13].

Decellularized human amnion (DAM)

Patients were recruited for this study with informed written consent using a protocol approved by the Ethical Committee of the District of Zurich (study Stv22/2006). Fetal membranes were collected immediately after birth from healthy women undergoing elective caesarean section, being tested negative for HIV and hepatitis B, with uncomplicated pregnancies without PPROM, signs of infection including no streptococcus B in their vaginal smear, or chromosomal abnormalities. Amnion was separated from chorion by blunt dissection and amnion decellularization performed under sterile conditions as described before[6]. Briefly, cells were removed by different enzymatic treatments and mechanical scraping. The resulting decellularized membrane was cut to 1cm x 1cm pieces and stored in sterile PBS until their surgical use.

Displaying the gelation speed of mussel glue *in vitro*

In order to display the gelation speed of the glue, 30µl drops of glue were dispensed in water immediately after mixing the glue components and every five seconds thereafter. Photographs were taken with a digital camera.

Surgical procedure

Twenty-four time-dated pregnant New Zealand rabbits were operated at 23 days of gestation. The rabbits were premedicated with intramuscular injection of ketamine 25 mg/kg (Ketamin 1000; CEVA sante animale, Libourne, France), xylazine 6 mg/kg (Vexylan; CEVA sante animale, Libourne, France), and buprenorphine, 0.02 mg/kg (Temgesic; Schering-Plough, Kenilworth, NJ), followed by anesthesia with isoflurane (1% to 1.5%) in oxygen at 1.5 L/min (Isoba Vet; Schering-Plough). Preoperative medroxyprogesterone acetate 9 mg/kg (Depo-Provera; Pharmacia-Upjohn, Puurs, Belgium) for tocolysis and Penicillin G 300,000 units IM (Penicillin; Continental Pharma, Brussels, Belgium) as prophylactic antibiotic were administered subcutaneously. The does were placed in a supine position and then shaved under continuous aspiration. After disinfection with povidone iodine (Isobetadine; Asta Medica, Brussels, Belgium), does were draped with sterile fields so that interventions on the uterus and the membranes were performed under sterile conditions using microsurgery instruments. The uterus was exposed through a midline abdominal incision. Gestational sacs were counted and numbered and the ovarian end sacs were randomly assigned to the treatment groups. One sac per rabbit, randomly chosen, served as positive control. The remaining sacs served as negative controls.

After dissection of the myometrium and chorion, enabling correct puncturing of the amniotic sac, amnion defects were created with a 14-gauge (2.1 mm) needle (Figure 2.1A). Treatment groups were as follows: mussel glue alone, mussel glue with DAM and fibrin glue with DAM. In the latter two groups DAM was first placed into the defect manually (Figure 2.1B) followed by immobilization with 100µl of fibrin or mussel glue (Figure 2.1C). The myometrial layers and the abdomen were closed by polypropylene 6-0 (Prolene; Ethicon, Neuchatel, Switzerland) sutures. After reposition of the uterus, the abdomen was closed in layers with polyglactin 2-0 (Vicryl, Ethicon) for the fascia and intracutaneous nylon 3-0 (Ethilon, Ethicon) for the skin. After recovery in the operating facility, the animals were returned to their cages and allowed free access to chow and water.

Eight days after implantation (at term) the does were euthanized with an i.v. bolus of a mixture of embutramide 200 mg, mebezonium 50 mg and tetracain hydrochloride 5 mg (T61®; Hoechst, Brussels, Belgium) for a second look hysterotomy. Presence of amniotic bands or skin defects of the fetuses were documented. The initial trauma sites on the fetal membranes were prepared for histology and immunohistochemistry by fixating the tissue in 4% formaldehyde for 24 hours.

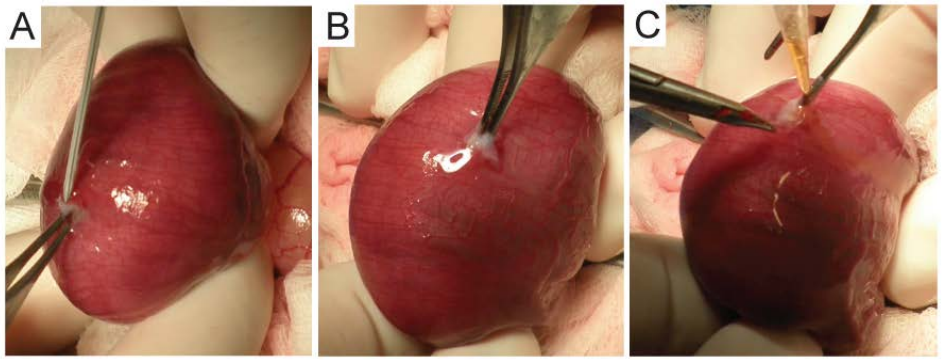

Figure 2.1 Surgical procedure A) Puncturing the membranes B) Placement of the DAM into the defect C) Application of mussel glue.

Evaluation of sealing and healing

Fetal survival was evaluated and membrane samples were taken only from sacs with living fetuses. Integrity of the sealed sacs was tested by injecting saline solution dyed with methylene blue using a 26-gauge needle through the membrane into the sacs on the opposite site of the defect. Four µm sections were made though the explanted and fixed fetal membrane samples and stained with hematoxillin-eosin (HE), monoclonal mouse anti-rabbit antibody for CD68 (Clone RAM11, Dako, Glostrup, Denmark) indicating macrophages, monoclonal mouse anti-vimentin antibody (Clone V9, Dako, Glostrup, Denmark) staining primarily cells of mesenchymal lineage and monoclonal mouse anti-cytokeratin antibody (Clone MNF116, Dako, Glostrup, Denmark) marking cells of epithelial origin. Primary antibodies were detected using horseradish peroxidase conjugated polyclonal goat anti-mouse immunoglobulins (Clone P0447, Dako, Glostrup, Denmark).

Image analysis

Images were recorded using the KS400 image analysis system (Carl Zeiss AG, Jena, Germany) linked to a Zeiss Axioskop microscope 50 equipped with a digital camera (Axio Cam MRc5, Carl Zeiss AG, Jena, Germany). Number of macrophages per tissue section area was determined by counting the CD68 positive cells in three locations in the proximity of the entry site. Images through the membrane were captured using a 10× objective and assembled into one image to avoid counting the cells in overlapping area twice and cell numbers were divided by the area of tissue determined using ImageJ[14].

Statistics

Results are presented as median (IQR). Statistical analysis was performed using the Kruskall-Wallis test with Dunn's post hoc test using SPSS plus 20.0 for Windows XP (SPSS, Inc, Chicago, IL). Significance level was set to $P < 0.05$.

RESULTS

Gelation speed of mussel glue

Immediately after mixing the cPEG solution with sodium periodate, the glue is still dispersing in water. Immediately thereafter it becomes increasingly viscous and after only 30 seconds polymerization has progressed such that the mussel glue material cannot be pipetted or dissolved in water (Figure 2.2).

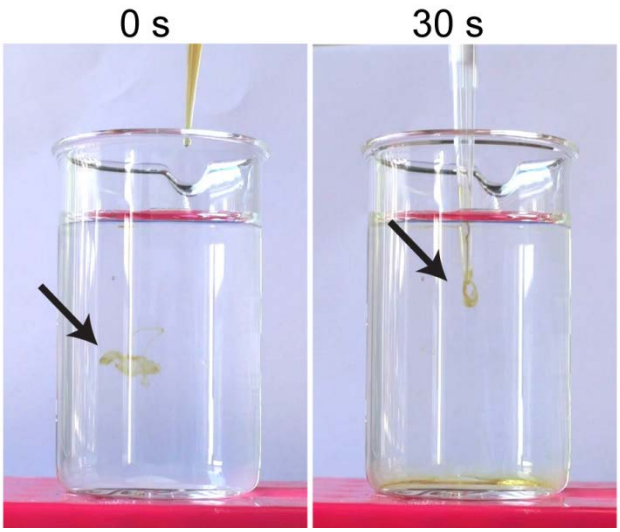

Figure 2.2 Gelation of mussel glue. Immediately after initiating polymerization the glue is still liquid and disperses in water, but remains attached to the pipet tip after 30 seconds.

Fetal survival, skin defects, amniotic bands and macroscopic evaluation of sealing

All operated does survived the operation and none of them miscarried, though all fetuses of one doe were found dead at the time of explantation, which most likely was due to a large thyroid growth present at the time of operation and euthanasia. No fetuses were found herniating into the maternal abdomen at the second laparotomy. No amniotic bands or intra-amniotic adhesions could be seen after opening the gestational sacs and the surviving fetuses did not show any signs of skin defects (Table 2.1). Fetal survival was comparable for all treatment groups as well regarding the negative controls (Table 2.1). Membrane integrity was higher in all treatment groups compared to the positive control (Table 2.1).

Table 2.1 Outcome of different treatment groups

	Neg. control	Pos. control	MG+DAM	MG	FG
Alive	39	4	8	6	6
Dead	54	7	2	4	9
Survival rate	42%	36%	80%	60%	40%
Leaky sac (fetus alive)	NA	3/4 (75%)	2/8 (25%)	2/6 (33%)	0/6 (0%)
Amnion bands	No	No	No	No	No
Skin defects	No	No	No	No	No

Histological evaluation

Both sealants and scaffold could be identified from all explanted tissues processed for histological evaluation. From HE stained sections it could be seen that mussel glue was always tightly attached to the surrounding tissue or scaffold whereas fibrin glue in half of the samples appeared to be mainly physically lodged into the defect without an adhesive interface with the tissue (Figure 2.3A). None of the explanted tissues sealed with either of the glues displayed considerable tissue remodelling or anatomical restoration of the puncture defect.

Mussel glue displayed good tissue adhesion, yet cellular infiltration into it was absent (Figure 2.3A and B). The DAM on the other hand had cells residing in it (Figure 2.3B). Cells interacting with the edges of mussel glue and inside the DAM were shown to be mainly mesenchymal by positive vimentin staining (Figure 2.3C). Cytokeratin positive epithelial cells were in some of the mussel glue samples found as small clusters around the glue, but not as a continuous lining on top of it (Figure 2.3D).

For the fibrin glue samples, HE staining displayed cellular infiltration into the fibrin and into the DAM in the samples where fibrin was attached to the tissue (Figure 2.3B), but not when adhesion was absent (Figure 2.3A). The phenotype of the invading cells was mainly mesenchymal (Figure 2.3C), but epithelial cells were also found lining the scaffold and gluing material (Figure 2.3D).

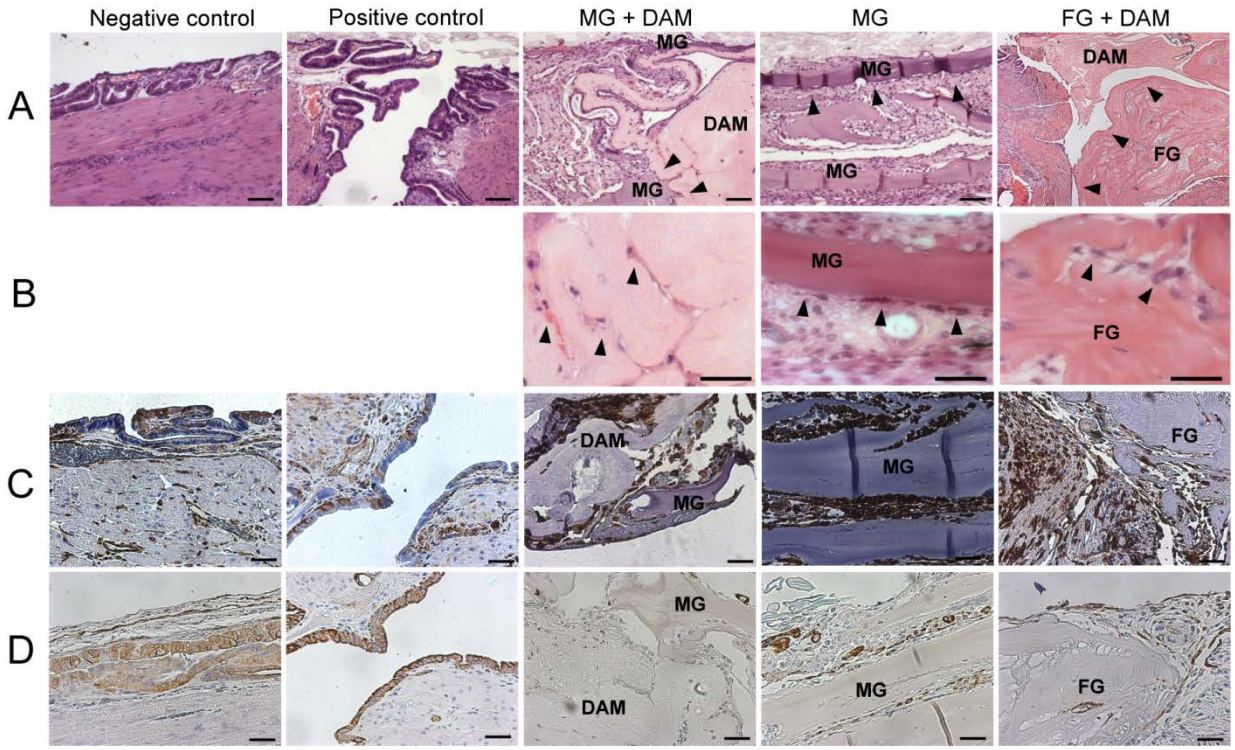

Figure 2.3 Representative sections from plugging sites. A) Hematoxillin-Eosin (HE) staining showing that mussel glue (MG) is tightly attached to surrounding tissue and to DAM, whereas fibrin is less attached (black arrows indicate the interface of gluing materials

CD68+ cells indicating macrophage phenotype were present in all treatment groups as well as in the positive control group (Figure 2.4A). They were mainly found as clusters at the immediate vicinity of the plugging materials and were absent at other parts of the samples. Number of CD68+ cells was higher in the case of the mussel mimetic sealant, but only significant in the group without the DAM (Figure 2.4B).

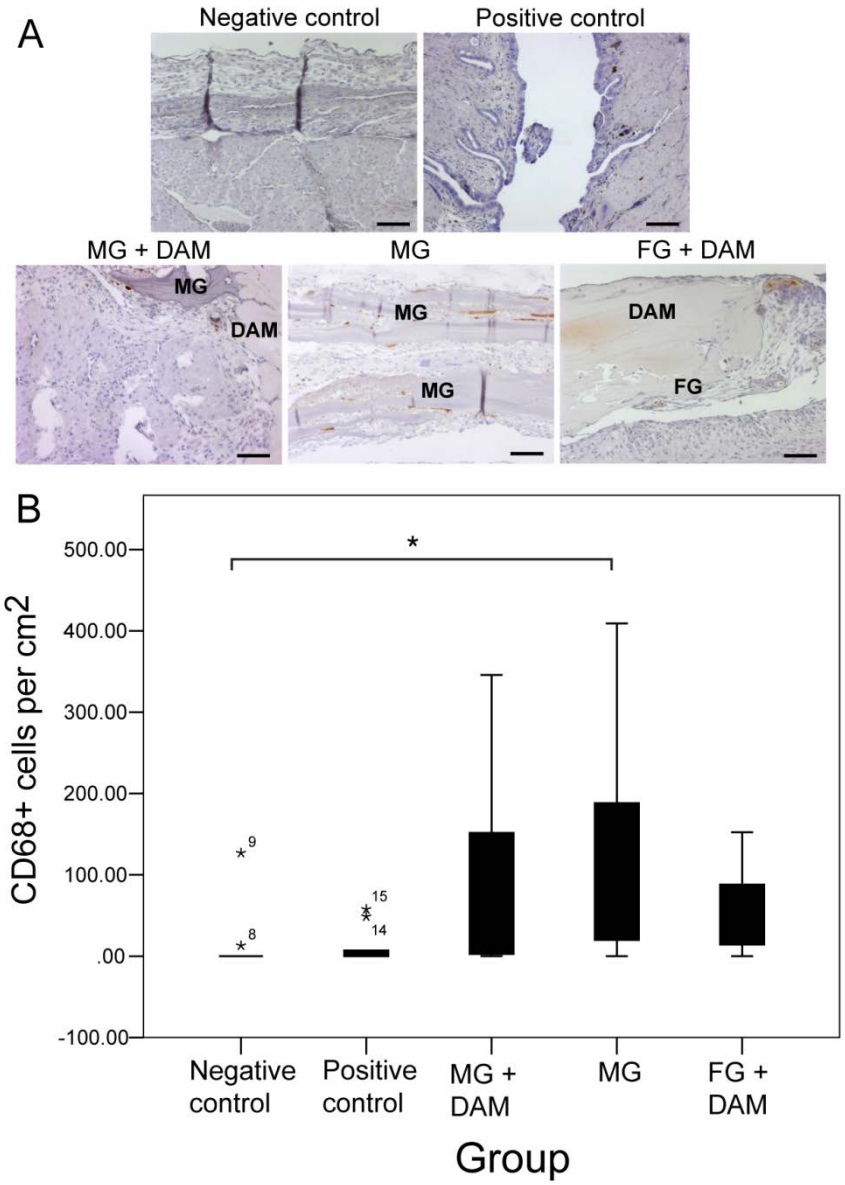

Figure 2.4 CD68+ macrophages at the plugging sites. A) Representative images showing clusters of macrophages in the proximity of the plugging materials. Scale bar 100µm. B) Number of CD68+ per area of tissue section at plugging site**.**

DISCUSSION

Previously characterized mussel mimetic sealant was for the first time tested *in vivo* in the context of sealing iatrogenic fetal membrane defects. Commercially available fibrin sealant has previously been used for fetal membrane sealing in rabbits[6] and was thus used as a "golden standard". Both glues were tested together with a decellularized amnion plug, as such a plugging strategy could provide both initial sealing as well as a provisional scaffold for the initiation of healing and result in successful short

and long term repair of the punctured membranes. In accordance to *in vitro* and *ex vivo* sealing and toxicity experiments done previously[9], mussel mimetic sealant also displayed good adhesive properties *in vivo,* without causing any adverse side effects. Fetal survival in our experiments was relatively low compared to previously published studies using the same animal model[5, 6], but it could not be attributed to any of treatments as fetal death occurred similarly in the untreated group.

Based on this short term *in vivo* evaluation, mussel glue's sealing capability was comparable to that of fibrin glue. *In vitro* and *ex vivo* testing has shown that mussel glue has better mechanical and adhesive properties than fibrin glue and is also less susceptible to proteolytic degradation[10]. This initial short term investigation of the sealants performance *in vivo* in the context of sealing fetal membrane defects showed that the mussel glue tightly adheres to fetal membranes and persist at least over the one week evaluation period.

Absence of cellular infiltration into the mussel glue indicates that the glue could remain stable for a relatively long period of time, supporting the membrane integrity during the relatively slower process healing via a cell instructive plug. Providing that the original adhesion to the membranes had taken place, which was only true for half of the sealed defects, cell infiltration in fibrin glue demonstrated that cells readily degrade it *in vivo*. Fibrin glue was previously shown to lack long term proteolytic stability *in vitro*[10] supporting these *in vivo* observations.

Mussel glue moderately increased macrophage recruitment to the gluing site compared to fibrin glue. However, due to the short duration of implantation we could not conclude whether this would eventually lead to chronic inflammation or was part of a constructive remodelling process. CD68 is a pan macrophage marker and antibodies for rabbit type M1 and M2 type macrophages[15], which would allow a more detailed, investigation, are not currently available. As an elevated inflammatory reaction could be expected due to the synthetic nature of mussel glue compared to fibrin, careful monitoring of such reactions in larger animal models (sheep) is required.

In conclusion, the performance of mussel glue during one week was comparable to the commercial fibrin glue. As other studies have pointed out mussel glue's better sealing capability, mechanical properties and proteolytic stability, it is a strong candidate to clinically applicable solution to iPPROM. Nevertheless, longer testing periods in larger animal model (sheep) are needed to determine the ultimate potential of mussel glue.

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CHAPTER 3 Engineered cell instructive matrices for fetal membrane healing

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ABSTRACT

Iatrogenic preterm prelabour rupture of fetal membranes (iPPROM) occurs in 6-45% of the cases after fetoscopic procedures posing a significant threat to fetal survival and well-being. Number of available diagnostic and therapeutic fetal interventions is increasing and thus developing treatment options for iPPROM is growing more important than ever before. Fetal membranes exhibit very restricted regeneration and little is known about factors which might modulate their healing potential, rendering various materials and strategies to seal or heal fetal membranes pursued over the past decades relatively fruitless. Additionally, biocompatible materials with tunable *in vivo* stability and mechanical and biological properties have not been available. Using poly(ethylene glycol) (PEG)-based biomimetic matrices we provide evidence that upon presentation of appropriate biological cues in 3D, mesenchymal progenitor cells from amnion can be mobilized, induced to proliferate and supported in maintaining their native extracellular matrix production, thus creating a suitable environment for healing to take place. These data suggest that engineering materials with defined mechanical and biochemical properties and the ability to present migration and proliferation inducing factors, such as PDGF, bFGF, or EGF could be key in resolving the clinical problem of iPPROM and allowing the field of fetal surgery to move forward.

Keywords: Fetal membrane, healing, growth factor, PEG, synthetic matrix, PPROM, amnion, 3D cell migration

INTRODUCTION

Iatrogenic preterm prelabour rupture of fetal membranes (iPPROM) occurring in 6-45% of the cases after fetoscopic interventions is a serious complication associated with fetal morbidity and mortality [1]. It is linked with a range of serious complications including respiratory distress syndrome (RDS), cerebral palsy, blindness, deafness and necrotizing enterocolitis [2]. After decades of research iPPROM has up to date remained an unsolved clinical problem affecting millions pregnancies worldwide. Compromising the expected benefits of any intrauterine intervention, it is a serious complication for prenatal diagnostics and fetal surgery and a major obstacle for the further development of the field [3].

Human fetal membrane is a bilayer structure enclosing the amniotic cavity, consisting of a stronger inner layer, the amnion, and a more pliant outer layer, the chorion. Amnion is made of a stromal layer, a thick basement membrane and a single epithelium facing the amniotic cavity [4]. Because fetal membranes are very poorly vascularized, the lack of a typical wound healing response including inflammation, scar formation, and tissue regeneration as described in the skin [5] and many other organs is not to be expected. As shown by clinical experience and animal studies, the healing potential of the membranes after fetoscopic intervention is very limited [6]. The lack of healing response is not completely understood, but could be explained by excessive matrix remodeling by matrix metalloproteinases (MMPs) [7], or most probably by the lack of a provisional cell guiding matrix, containing appropriate healing signals, normally provided by fibrin plugs.

Based on the hypothesis that cell instructive scaffolds can induce a biological repair cascade, decellularized human amnion membranes, containing all the native matrix signals, have been evaluated both *in vitro* and *in vivo* in a rabbit mid-gestational model [8][9]. Porcine small intestine derived matrices [10], collagen or gelatin sponges [11][12][13], collagen slurry [14] and matrigel [13] have also been tested in *in vivo* models [8][15][16] with variable success. Fibrin sealants have been evaluated *ex vivo* as well as in animal models and in patients [11][17]. Some of these repair strategies relying on naturally occurring materials have shown potential for iPPROM sealing, but due to limited control over critical materials properties such as proteolytic stability and biological functionality, they could not be further optimized.

Fetal membranes have been hypothesized to harbor stem or progenitor cell populations as remnants of embryogenesis [18]. Several groups have investigated the potential of mesenchymal cells isolated from human amnion and shown that these cells can differentiate into various lineages [19][20]. If amnion indeed contains a progenitor cell component, stimulating these cells with proper signals could initiate the healing of the punctured membranes. Thus, identification of materials and biological signals able to control and promote amnion cell recruitment, proliferation and extracellular matrix production would be of great clinical significance.

We have recently described modular designed by transglutaminase factor XIII (FXIII) cross-linked poly(ethylene glycol)(PEG)-based biomimetic hydrogels [21][22] (named TG-PEG hydrogels) tailorable in terms of stiffness, proteolytic stability, and presentation of cell adhesion ligands and growth factors. These TG-PEG hydrogels, when formulated with low stiffness (30-250 Pa), MMP degradable cross-links, and 50 μM RGD were shown to allow efficient migration of mesenchymal cells (osteoblasts and fibroblasts [23]). Additionally, the TG-PEG hydrogels permitted the covalent immobilization of peptides, growth factors and other bioactive molecules that contained a short α2-plasmin inhibitor derived FXIII transglutaminase glutamine acceptor domain (Gln; NQEQVSPL) [22].
Taking advantage of this proteolytically stable, mesenchymal cell migration compatible hydrogels we herein describe the screening for soluble factors promoting and directing the healing of fetal membranes using *in vitro* and *ex vivo* models. We have established factors, which can be employed for the *in vitro* mobilization of human amnion mesenchymal cells (hAMCs) from fetal membrane tissues and support both their proliferation and the formation of cellular networks. This knowledge together with available growth factor immobilization and release strategies [22][24] will provide the basis for the producing cell-instructive microenvironments for healing of fetal membranes.

MATERIALS AND METHODS

Cell isolation, culture and characterization

Mesenchymal and epithelial cells were isolated from human amniotic membranes as described previously [18] and characterized using flow cytometry (detailed description in supplementary information). All experiments were repeated on cells from three different donors.

Formation of microtissues

Microtissues consisting of 1000 cells were formed by pipetting 30 μL drops of cells in culture medium containing 20% methylcellulose (Sigma Aldrich, St Louis, MO, USA) on non-adhesive bacterial culture dishes which were then turned upside down. Cells in hanging drops were allowed to aggregate overnight at 37°C and the resulting microtissues were collected by washing the lid with 1% bovine serum albumin (BSA, Applichem) in PBS. Microtissue suspension was centrifuged for 5 min 600 rpm and resuspended in cell culture medium.

PEG hydrogel formation

TG-PEG hydrogel formation was performed as previously described [21][22]. Briefly, functionalization of eight-arm PEG-vinylsulfone (PEG-VS) with the FXIII substrate peptides glutamine acceptor substrate or lysine donor substrate containing a MMP-sensitive linker resulted in n-PEG-Gln or n-PEG-$MMP_{sensitive}$-Lys monomers [21][22]. Covalently cross-linked hydrogels were formed by the addition of 10 U ml^{-1} of thrombin-activated factor XIIIa to a tris buffered saline (50 mM, pH 7.6) solution

containing besides a stoichiometrically balanced amount of n-PEG-$MMP_{sensitive}$Lys and n-PEG-Gln 50 mM calcium chloride and 50uM Gln-RGD. For hydrogels with containing covalently bound a_2PI_{1-8}-PDGF-BB, the growth factor was added to the hydrogel mass prior to the onset of gel formation. Gelation occurred within a few minutes at room temperature, but the cross-linking reaction was allowed to further proceed for 30 min at 37 °C in a humidified incubator.

Cell, microtissue and tissue encapsulation into hydrogels

Suspension containing either 6.5 x 10^4 cells mL^{-1}, microtissues or small pieces (0.5 x 0.5 mm) of homogenized tissue in culture medium were added to the gel mass right before FXIIIa. After the addition of FXIIIa, 20 µL gel drops were sandwiched between two hydrophobic glass slides separated by spacers (approximately 1 mm), which were manually rotated for the first 5 minutes of gel polymerization to avoid cell or tissue sedimentation into the bottom of the gel. After polymerization, gels were released and immersed in culture medium.

Analysis of 3D cell migration

For migration studies, gels were glued to the bottom of cell culture wells with 5% PEG gel formulation and equilibrated in culture medium for 2 hours before starting the time lapse acquisition. In order to study collective 3D migration and screen for migration inducing factors, microtissues were encapsulated in hydrogels. Chemotactic effect of several factors reported to act as chemoattractant to mesenchymal cells [25][26][27][28][29], including basic fibroblast growth factor (bFGF, *Peprotech, Rocky Hill, NJ, USA*), epidermal growth factor (EGF, Sigma, St Louis, MO, USA), platelet derived growth factor (PDGF, Peprotech), transforming growth factor beta (TGF-β, Peprotech), tumour necrosis factor alpha (TNF-α, Biosource, Camerillo, CA, USA), insulin like growth factor two (IGF-2, Peprotech), stromal derived factor one (SDF-1, Peprotech), interleukin-6 (IL-6, R&D Systems, Minneapolis, MN, USA), vascular endothelial growth factor (VEGF, Peprotech), hepatocyte growth factor (HGF, Peprotech) was tested by adding them individually or in combinations into the medium (all 100 ng/ml).

In experiments directed towards studying growth factor induced migration mechanisms, broad spectrum MMP inhibitor GM6001 and Rho associated protein kinase (ROCK) inhibitor Y-27632 (both from Calbiochem) were added 1 hour prior to the addition of growth factors. Inhibitors were used at concentrations 50 µM (GM6001) and 10 µM (Y-27632) recommended by the manufacturer and described in literature [23].

Migration was visualized using time lapse microscopy (images captured every 10 minutes up to 48 hours). Collective migration out of microtissues was quantified by determining the area covered by the migrated cells from still images after 48 hours and 5 days using ImageJ [30]. Mean migration speed of single dispersed encapsulated cells was determined from the resulting movies using MOSAIC particle tracker plugin for ImageJ [31].

Analysis of cell viability, metabolic activity and proliferation

Cell viability in 3D hydrogel cultures after one week of growth factor stimulation was examined by LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR, USA). Briefly, ethidium homodimer-1 and calcein AM from the kit were diluted in 1:1000 ratio in culture medium and gels were stained with this solution for 10 min in cell culture incubator before imaging.

DNA content was measured using CyQUANT® NF Cell Proliferation Assay Kit (Invitrogen). After culture period of 0, 2, 5 or 10 days the gels were rinsed with PBS and stored at -80ºC until analysis. After thawing gels were first digested with Collagenase A (Roche, 2mg/ml in PBS) and the released cells were then stained with the CyQUANT dye for one hour at 37ºC. Fluorescence was then immediately measured at 520 nm using a microplate reader (Synergy HT, BioTek, Winooski, VT, USA).

Metabolic activity of the hydrogel encapsulated and growth factor stimulated cells was evaluated by WST-1 assay (Roche, Basel, Switzerland) after 48 hours, 5 days and 10 days of culture. Gels were transferred to new wells to exclude possible cells growing in the bottom of the well. WST-1 reagent was mixed with medium in 1:20 ratio and this mixture was added onto the gels. Medium was sampled every hour and the colour change determined by spectrophotometer using 450 nm wavelength. The values obtained after 3 hour incubation were used for evaluation.

RNA extraction, cDNA synthesis and real time PCR analysis

Quantitative real-time polymerase chain reaction was used to measure gene expression levels of collagen I, collagen III and elastin in hAMC grown in different conditions. Cells were either used immediately after isolation, after culture on plastic or after culture in 3D hydrogels with different growth factors. RNA extraction was performed using RNeasy Micro Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. Prior to this, PEG gels were washed with PBS for 5 min and digested with Collagenase A (Roche, 2mg/ml in PBS) on ice for 1 hour. RNA was reverse transcribed to cDNA using Sensiscript Reverse Transcription Kit (Qiagen) and real time PCR was performed with gene specific primers (QuantiTect Primer Assays, Qiagen) and QuantiTect SYBR green PCR kit (Qiagen) using iCycler real-time PCR machine (Bio-Rad, Richmond, CA, USA). Data was normalized to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and freshly isolated, non-cultured cells (ΔΔCt method).

Immunocytochemistry and microscopy

Samples were fixed with 4% paraformaldehyde for 20 min followed by 5 min incubation in 0.1 M glycine and two washes with PBS after which they were permeabilized and blocked with 1% BSA and 0.2% Triton X-100 in PBS for 60 min before staining. F-actin was stained with Phalloidin-Alexa fluor 546 (Invitrogen, Carlsbad, CA, USA) in PBS with 1% BSA (Bovine serum albumin) overnight at 4˚C. For immunostainings, following primary antibodies were used at 1:100: mouse monoclonal anti-collagen I (Abcam, Cambridge, MA, USA), mouse monoclonal anti-elastin (Abcam), rabbit polyclonal anti-collagen

III (Abcam) and mouse monoclonal anti-paxillin (BD Pharmingen). DyLight™ 488 Goat anti-mouse IgG (Biolegend) or DyLight™ 649 Donkey anti-rabbit IgG (Biolegend) were used as secondary antibodies at 1:200. After washing the samples three times in PBS, cell nuclei were stained with 5 ng/mL DAPI (40,6-diamidino-2-phenylindole) (Sigma Aldrich) in PBS for 10 min at room temperature.

Sample evaluation was performed by either confocal laser scanning microscopy (Leica TCS SP5) or epifluorescence microscope (BM550B, Leica Microsystems, Germany). Time lapse movies were recorded using the KS400 image analysis system (Carl Zeiss AG, Jena, Germany) linked to a Zeiss Axioskop microscope 50 equipped with a digital camera (Axio Cam MRc5, Carl Zeiss AG, Jena, Germany).

Statistical analysis

Results are presented as mean ± SD. Data was treated as non-parametrical and statistical analysis was performed using the Kruskall-Wallis test with Dunn's post hoc test using SPSS plus 20.0 for Windows XP (SPSS, Inc, Chicago, IL). Significance level was set to $P < 0.05$.

RESULTS

Growth factor mediated mobilization of hAMCs from microtissues

To test the potential of various growth factors to promote the mobilization of amnion tissue cells, model microtissues consisting of hAMC (expressing characteristic mesenchymal stem cell markers CD90, CD73, CD44, CD105, CD146 and CD166, for detailed information refer to supplementary information and Supplementary Figure 3.1) were formed and embedded in MMP-degradable PEG-based biomimetic matrices which contained the prototypic integrin ligand RGD. As observed for other mesenchymal cells, the migration of hAMCs out of the microtissue was almost instantaneously initiated (6h) after stimulation with PDGF resulting in a radial invasion of the hydrogel (Fig. 3.1A). In contrast, unstimulated cells remained within the microtissue. Time-lapse movies demonstrate that depending on the growth factor (PDGF, bFGF, EGF, TGF-β) migration speed varies substantially (Supplementary movies S1 to S5, available online). Quantification of the migration distances after 48 hours displayed significantly increased migration from the micro-tissues in response to PDGF (4-fold), bFGF (2-fold) and EGF (1.75-fold) when compared to unstimulated cells (Fig. 3.1B). Whereas TGF-β slightly induced the mobilization of cells, none of the other tested factors showed any effect. After 5 days the migration distances further increased in the case of PDGF (6-fold), bFGF (3.5-fold), EGF (2-fold) and TGF-β (Fig. 3.1A). Some migration could also be observed in the case of TNF-α and IGF-2. Time-lapse and confocal microscopy evaluations of the migrated cells demonstrated not only large variation in the onset and distance of migration, but also indicated that migration behaviour and mechanism are distinct between the factors (Fig. 3.1C and supplementary videos available online). For example, PDGF stimulated cells migrated as small single cells and subsequently formed a dense cellular network whereas bFGF

induced an elongated morphology and formation of chains of single cells. In contrast EGF and TGF-β stimulated cells migrated as multicellular protrusions indicating that hAMC in response to different factors under *in vitro* conditions are able to use distinct migratory mechanisms.

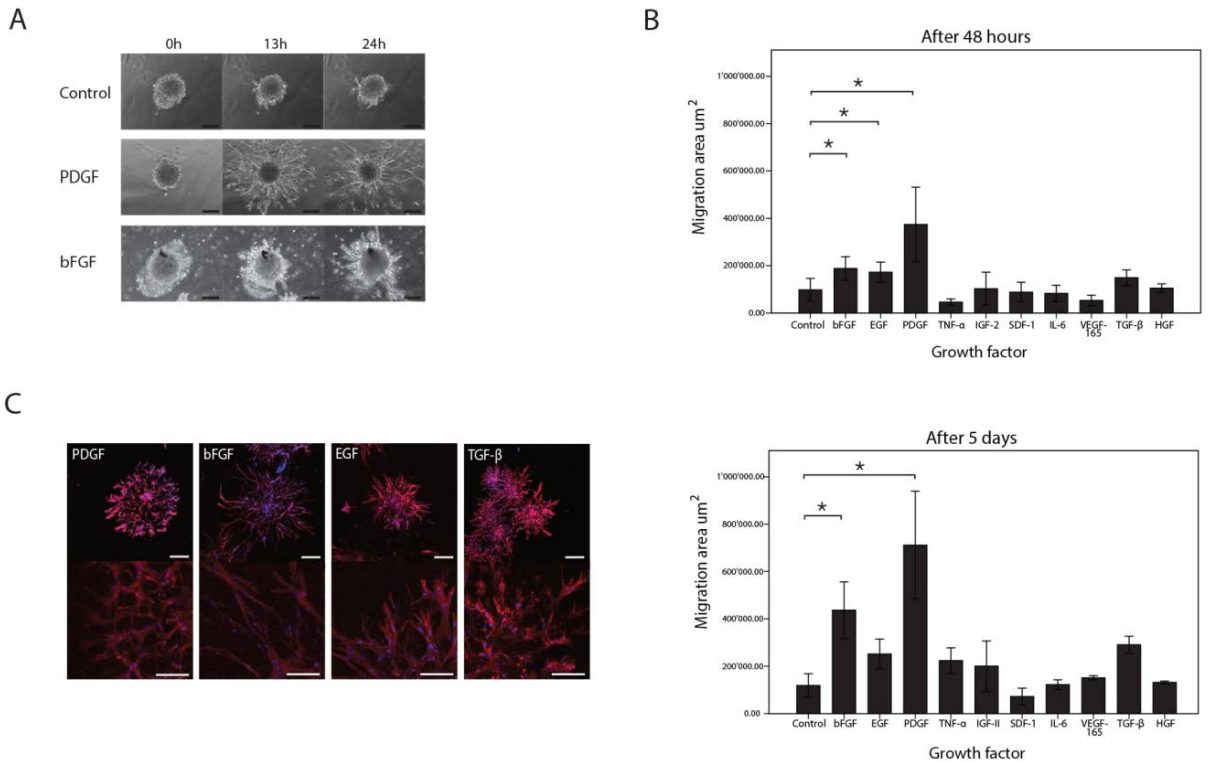


Figure 3.1 Screen for hAMC migration inducing factors. Microtissues consisting of hAMCs were embedded in PEG-based biomimetic hydrogels containing 50 µM RGD and stimulated by the addition of growth factors to the cell culture medium. (**A**) Migration time course in response to no growth factor, PDGF or bFGF during the first 24 hours. Scale bars, 100 µm. (**B**) Quantification of the migration response to different factors after 48 hours and 5 days. Data are means ± SD (n = 6) (**C**) Confocal images showing the morphological differences of the migrating cells at low (upper row) and high (lower row) magnification in response to PDGF, bFGF, EGF and TGF-β. Actin staining in red and DAPI for nuclei in blue. Scale bars, 200 µm (low magnification) and 100 µm (high magnification).

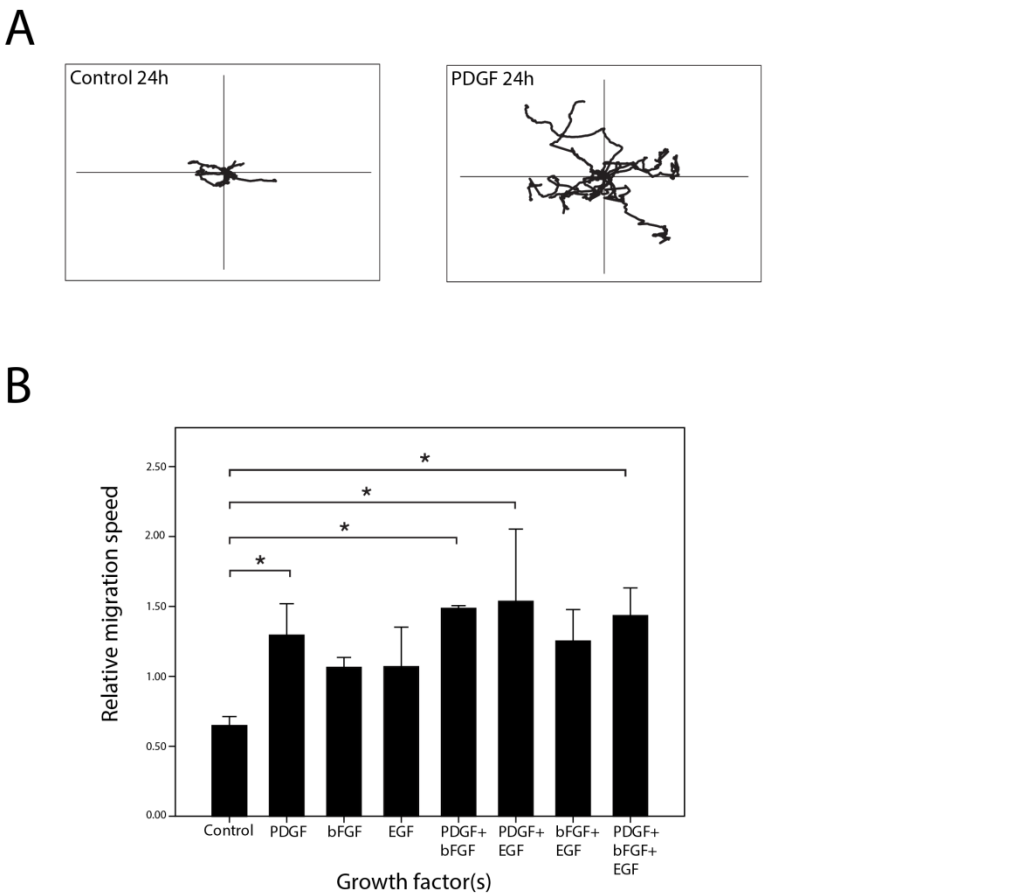


Figure 3.2 Migration of single hAMC in response to growth factors. 6.5x104 hAMCs/ml were encapsulated in PEG-based biomimetic hydrogels containing 50 µM RGD and stimulated by the addition of growth factors to the cell culture medium. (A) Examples of tracks produced by unstimulated and PDGF stimulated hAMC during 24 hours displaying the increase in cell motility. (B) Quantification of the migration response by speed of the migrating cells.

Evaluation of hAMC migration mode

To determine the influence of growth factors on hAMC migration behaviour cells were encapsulated in hydrogels at low seeding densities and the migration paths of individual cells could be followed by time-lapse imaging (Fig. 3.2A). In the initial 24 hours of treatment, 3D migration speed of single cells was found to be stimulated with PDGF, bFGF and EGF as compared to the control without growth factor though the difference to unstimulated cells was only significant for PDGF (Fig. 3.2B). Combining PDGF with bFGF, EGF or both also resulted in significant increase in migration speed compared to unstimulated cells, but not to PDGF alone. Effect of different growth factor combinations on migration speed was additive. Comparison of representative migration tracks of unstimulated and PDGF treated cells also demonstrates the increase in migratory activity (Fig. 3.2B).

Since for stimulation with PDGF both cell morphology and individual cells migrating was typical for mesenchymal mode [32], and for bFGF and EGF stimulation a multicellular migration mechanism was observed, the contribution of Rho/ROCK activity to migration was evaluated. Interestingly, upon treatment with the ROCK inhibitor Y-27632, PDGF stimulated hAMCs (from microtissues or single dispersed cells) adapted a more elongated phenotype and migrated in a multicellular mode, comparable to the one observed under bFGF and EGF treatment (Fig. 3.3A). Surprisingly, the ROCK inhibitor Y-27632 did not inhibit but rather promoted migration of hAMCs from micro-tissues in all growth factor treated samples (Fig. 3.3A), demonstrating that Rho/Rock pathway modulates the observed migration processes. However, in cultures of single dispersed hAMCs Y-27632 inhibition a trend towards reduced migration speed of EGF and TGF-β treated cells and promoted migration of PDGF stimulated cells was observed.

The migration of mesenchymal cells in response to inflammatory cytokines was described to be regulated by the expression of matrix metalloproteinases (MMPs) [33][34]. To demonstrate that multicellular migration processes are dependent on MMP mediated degradation of the MMP-1 sensitive substrates employed, growth factor stimulated cells were treated with the broad spectrum MMP inhibitor GM6001 (Fig. 3.3B). For the growth factor stimulated migration of cells starting from encapsulated microtissues or from single dispersed cells, MMP degradation of the substrates was required. Thus, not only mesenchymal migration of individual cells, but also multicellular migration, possibly dependent on the leading cells, rely on substrate modifications by MMPs.

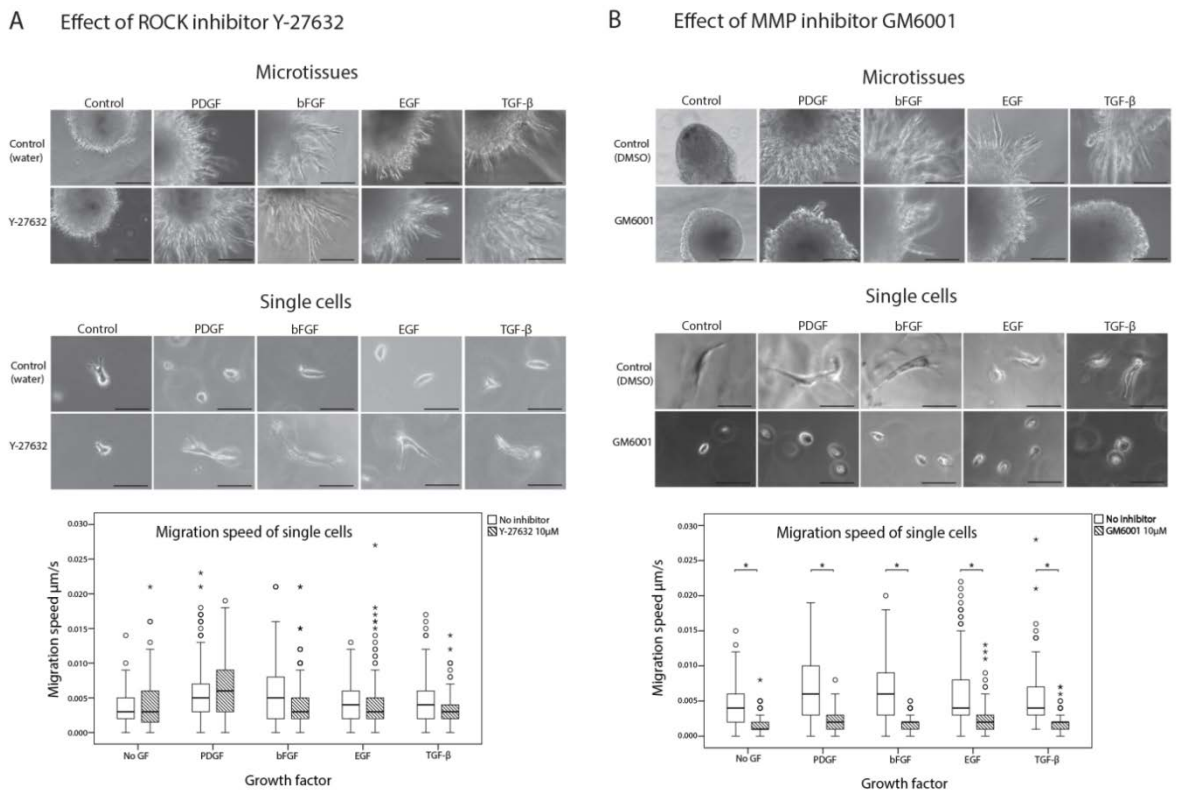


Figure 3.3 Inhibition of growth factor stimulated 3D migration of hAMCs by MMP-inhibitor (GM6001) and ROCK inhibitor (Y-27632). (A) Bright field images of hAMC migration out of microtissues and as single cells in the presence of Y-27632. Analysis of migration speed of growth factor stimulated cells subjected to Y-27632. (B) Bright field images of hAMC migration out of microtissues and as single cells in the presence of GM6001. Analysis of migration speed of growth factor stimulated cells subjected to GM6001. Scale bars, 100 µm (microtissues) and 200 µm (single cells).

Ex vivo mobilization of hAMCs from amnion tissue

Next we wanted to investigate whether the above identified migration inducing growth factors have the potential to mobilize cells from surgically injured fetal membranes into cell-instructive biomimetic PEG-hydrogels. Therefore 0.5mm x 0.5mm pieces of intact fetal membranes were encapsulated in the hydrogels and stimulated by application of soluble PDGF, bFGF, EGF or TGF-β. All factors could mobilize cells from intact amnion tissue into the PEG hydrogels whereas no cellular outgrowth could be seen in unstimulated control samples (Fig. 3.4). Morphological differences between growth factors similar to those observed before could also be seen, suggesting that the microtissue assay could accurately recapitulate the migratory behaviour of tissue residing cells. Interestingly, in addition to mesenchymal cells, EGF could induce the outgrowth of cyst like structures typical for epithelial cells in 3D [35] suggesting that EGF could also stimulate hAEC. Immobilized cells in all conditions displayed staining for paxillin in contrast to flat, inactivated cells remaining inside amnion pieces.

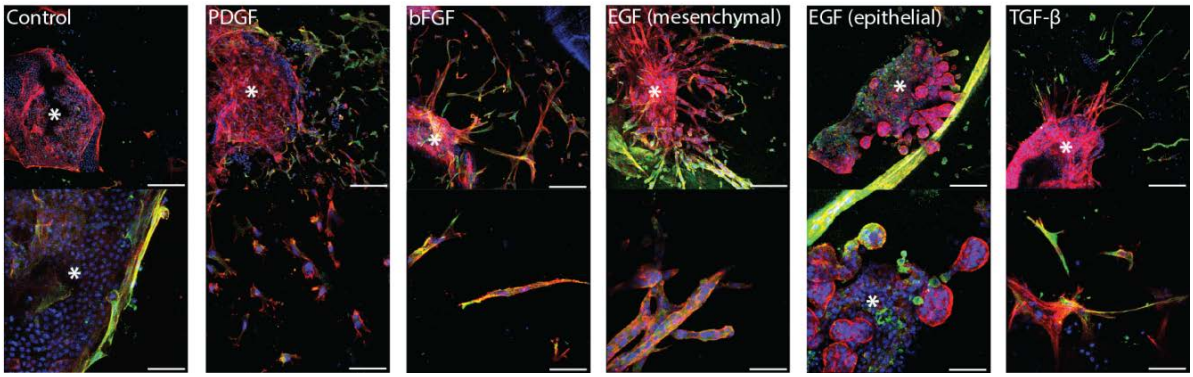


Figure 3.4 Migration of amnion cells out of amnion explants. 0.5mm x 0.5mm freshly harvested amnion pieces (indicated in the images by an asterisk) were embedded in PEG-based biomimetic hydrogels containing 50 µM RGD. Confocal images show the morphology of migrating cells in response to different factors at low (upper row) and high (lower row) magnification. Actin in red, paxillin in green and DAPI in blue. Scale bars, 500 µm (low magnification) and 100 µm (high magnification).

Influence of growth factor treatment on tissue formation

Since successful mobilization of cells is only one aspect of healing, we next evaluated the influence of the applied growth factors on viability, proliferation, and formation of cellular networks in long term cultures of hAMCs. The presence of mostly green stained cells in a live/dead assay of one week old hydrogel cultures showed that cells in all conditions had remained viable (Fig. 3.5A) verifying that the RGD containing hydrogel formulation is suitable for maintaining hAMC.

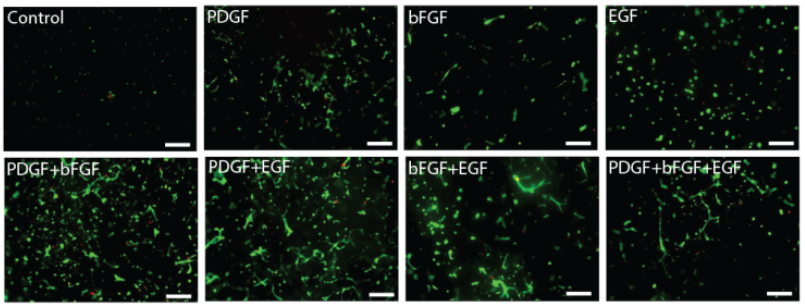
Cell proliferation as assayed by quantifying DNA content indicated a significant loss of viable cells during hydrogel encapsulation of the hAMCs. Values obtained at day 2 were not significantly different between the control group and the growth factor treated cultures indicating that growth factors had neither significantly contributed to cell survival nor proliferation in this initial culture phase. DNA amounts stayed almost constant for both controls and bFGF but increased for PDGF and EGF though the increase was only significant after 10 days of culture (Fig. 3.5B). These results suggest that all growth factors initially (2 days) promoted the 3D migration of hAMCs without significantly stimulating proliferation and only later proliferation supports the observed outgrowth of cells.

To support the data on DNA content, metabolic activity at different time points of hydrogel culture was also assayed. During the initial 48 hours of culture no differences could be seen between controls and growth factor stimulated cultures (Fig. 3.5C). While after 5 days of stimulation with growth factors only minor beneficial effect could be seen, after 10 days of culture the significantly increased metabolic activity in the PDGF (5.5-fold increase), PDGF and bFGF (5-fold increase) and PDGF and EGF (8.5-fold increase) stimulated cultures suggested increased cell numbers (Fig. 3.5C).

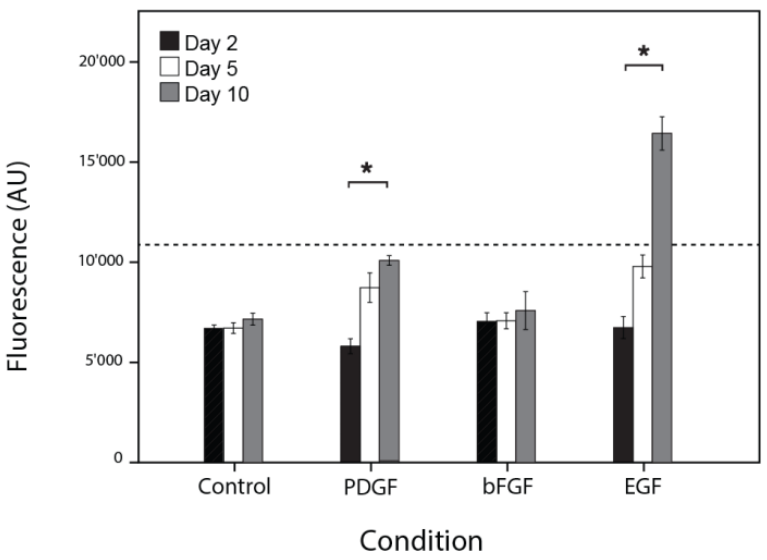
Additionally to the induction of proliferation in long term cultures, starting from single dispersed cells, stimulation with PDGF or bFGF alone influenced the spreading of the cells and induced the formation

of interconnected cellular networks (Fig. 3.5D). These effects were further enhanced by combined treatments with PDGF and EGF, bFGF and EGF, or PDGF, bFGF and EGF (Fig. 3.5D). In contrast, cells grown in the absence of additional growth factors spread only during the first days of culture, did not form cellular networks and acquired a rounded morphology after one week (Fig. 3.5D). These findings demonstrate that PDGF, bFGF and EGF in addition to their role in mobilizing hAMCs from fetal membranes can also stimulate the survival and proliferation of these cells.

A



B



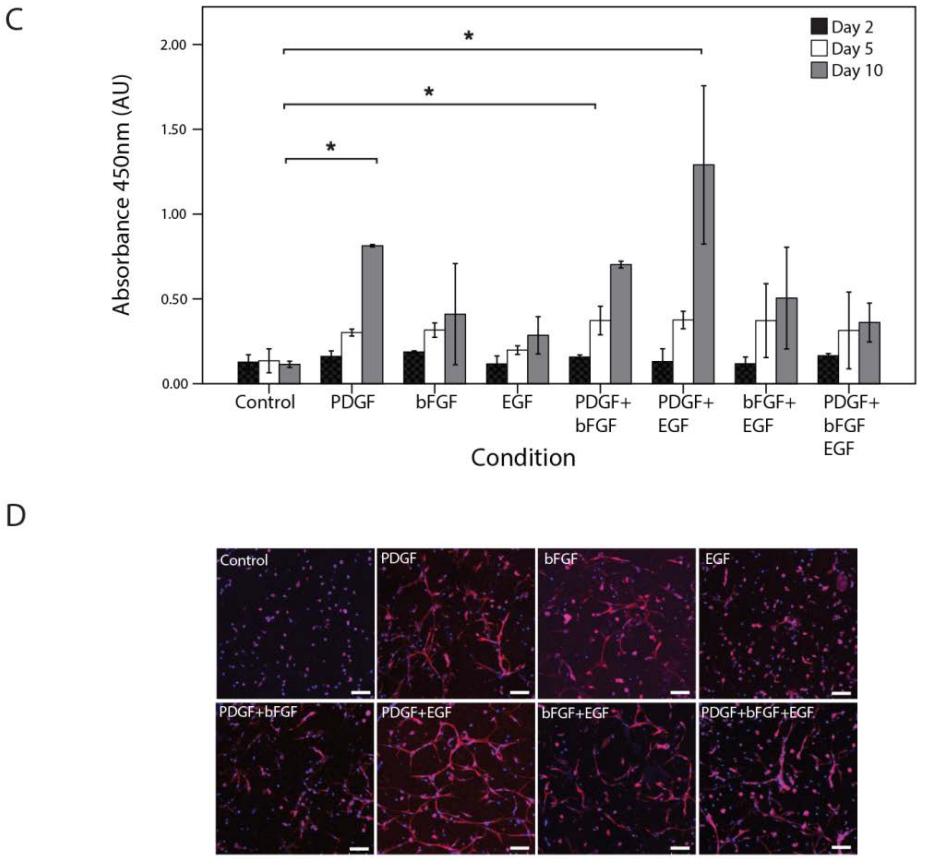


Figure 3.5 hAMC behavior in long term cultures. (A) Cells encapsulated at low density were cultured for one week in presence of growth factors and then stained for live (green) and dead (red) cells. Scale bars, 200 μm. (B) Quantification of metabolic activity in presence of different growth factors was quantified by determination of metabolic activity reflecting the number of cells at different time points. (C) Fluorescence intensity representing DNA content in hydrogels cultured for 2, 5 and 10 days in different conditions. Dotted line represents the DNA content immediately after gel formation. (D) Cell morphology in hydrogels after ten days of culture displaying growth factor stimulated spreading and network formation. Scale bars, 100 μm.

Influence of growth factor treatment on ECM production

The mechanical and functional integrity of a healing tissue in addition to the cellular components is strongly dependent on the formation of new ECM. In order to maintain the barrier function of the fetal membranes, remodelling of the provisional wound healing matrix must be in tune with the formation of collagens type I and III or elastin, the main membrane ECM constituents produced by hAMCs. To evaluate the effect of the culture conditions on the expression of these matrix components, mRNA levels of cells cultured for four weeks on tissue culture polystyrene (TCP), or in biomimetic matrices were compared by quantitative PCR (qPCR) (Fig. 3.6A). On TCP, collagen I expression was higher (1.6-

fold of freshly isolated cells) compared to freshly isolated cells whereas expression of collagen III and elastin was lower (0.24 and 0.17-fold). Interestingly, the situation was inversed for the cells cultured in the biomimetic hydrogels. Collagen I expression was lower (0.6-fold) but collagen III and elastin mRNA was found in higher amounts (0.4 and 0.98-fold).

None of the growth factors stimulating migration or proliferation could enhance ECM component expression, but some actually lowered it for all three proteins (data not shown). In order to mimic a realistic treatment situation, where cells would not be subjected to the migration and proliferation stimulating factors for longer than needed to reach sufficient cell numbers in the hydrogel plug we then investigated the effect of transient growth factor treatment. For this cells were stimulated for two weeks with all three growth factors PDGF, bFGF and EGF to induce migration and proliferation after which cells they were cultured in absence of the growth factors for another two weeks (Fig. 3.6B). There were no significant differences between cells cultured without growth factors for four weeks or cultured with growth factors for two weeks and another two weeks without them. This indicates that the negative impact of growth factors stimulation on ECM protein gene expression can be revoked upon their removal.

Both collagens and elastin could also be detected on protein level by immunostainings in cells cultured in hydrogels without growth factors after 4 weeks (Fig. 3.6C).

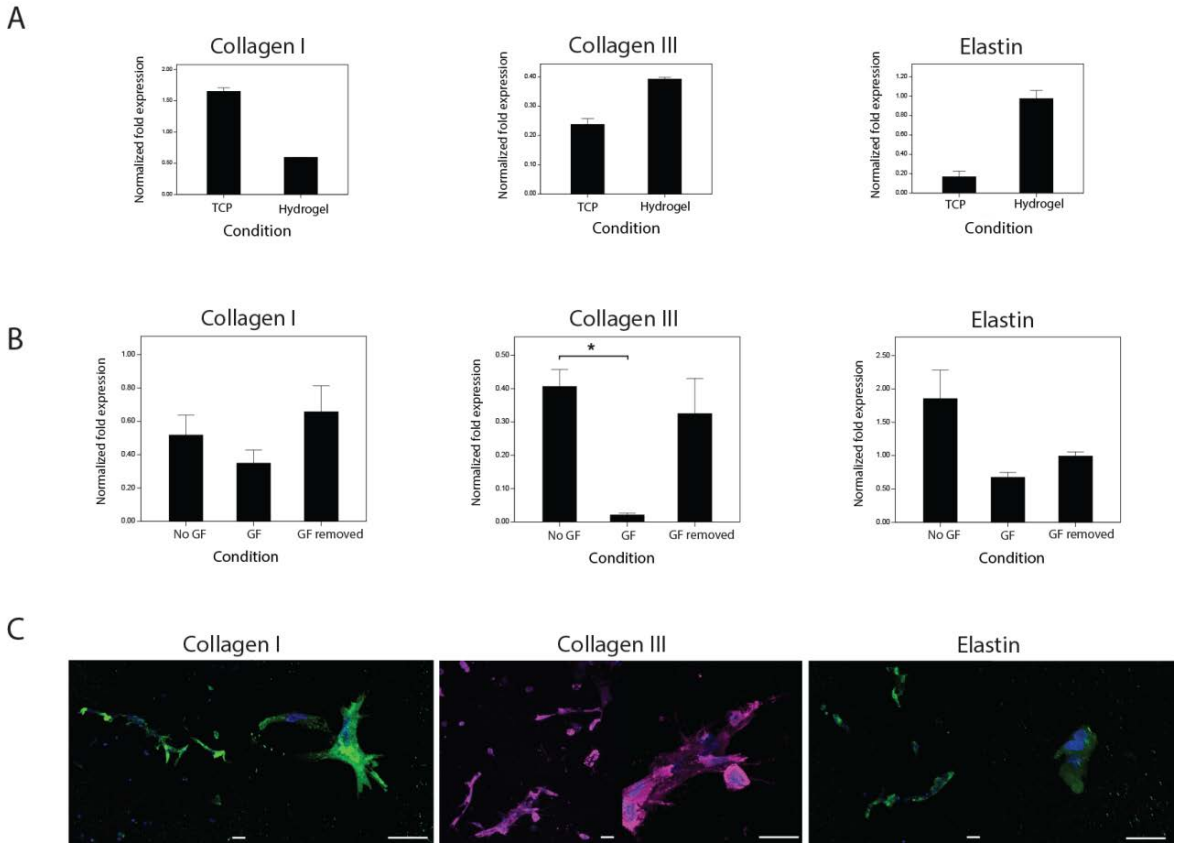


Figure 3.6 Production of amnion ECM components in cultured hAMC. (A) mRNA expression levels of collagen 1, collagen 3 and elastin in cells cultured on tissue culture plastic (TCP) or in PEG hydrogels for four weeks. (B) Expression levels of cells grown in PEG hydrogels with or without PDGF, bFGF and EGF (no GF or GF) and cells subjected to growth factors for the first two weeks followed by two weeks culture without growth factors (GF removed) (C) Immunostainings displaying collagens I and III and elastin in hAMC grown in PEG hydrogel for 4 weeks without additional growth factors. Scale bars, 100 µm (low magnification) and 50 µm (high magnification).

DISCUSSION

Factors promoting the mobilization of fetal membrane cells and biomaterials specifically tailored towards application in the amniotic cavity are critical for the development of healing strategies for iPPROM. Here we have employed synthetic, biomimetic matrices to develop *in vitro* and *ex vivo* fetal membrane healing models. We have identified growth factors which are promising candidates to promote the healing of surgically induced fetal membrane defects by mobilizing hAMCs from amnion and by supporting their proliferation and formation of cellular networks

Fetal membranes have a limited ability to heal causing surgically induced defects to persist throughout pregnancy [6]. A functional healing microenvironment should consist of a provisional matrix containing cell-instructive signals recruiting and directing tissue progenitor cells [36][37][38]. Due to

the absence of vascularization in the fetal membranes, it can be assumed that the appropriate milieu for healing established by the blood clot in other injured tissues cannot be established. Several studies have aimed at replacing this provisory guiding matrix with mostly biological materials having been pursued in animal models. Though some signs of healing have been observed in these experiments, both the stability of the employed matrices and the mobilization of cells were insufficient to establish functional and stable closure of the membranes [9][16].

Fetal membranes, directly evolving from the epiblast and the hypoblast during early embryonic development (Reviewed in [39]) have been described to contain cells within the epithelial and mesenchymal fractions which express typical stem cell surface markers, have large proliferation potential, and can give rise to multiple cell types [40][41]. Thus we hypothesized that in addition to the deployment of biomaterials with appropriate *in vivo* stability, the presentation of chemotactic signals would be sufficient to initiate the healing of fetal membranes by recruitment of progenitor cells with desirable healing capacity.

Previous attempts to study factors for the mobilization of progenitor cells from fetal membranes have been conflicted by the inherent proliferation and migration capacity in standard 2D culture conditions [42][43][44][45]. Thus *in vitro* models to study the behavior of amnion cells in 3D have been developed using collagen or fibrin gels [46][47]. Amnion mimicking constructs could successfully be assembled by embedding the hAMC in 3D fibrin or collagen matrix and seeding hAEC on top and the cells retained their vitality and natural morphology [47]. However, the materials properties of collagen proved to be unsuitable for maintaining these constructs for longer periods of time, as even in the presence of plasmin and MMP inhibitors, it experienced strong contraction by the mesenchymal cells. Recently, compressed collagen was used as a scaffold to create fetal membrane mimicking tissue *in vitro* [46]. Cells could be maintained in the gels and morphology was similar to the native tissue. Though these results are promising, the lack of flexibility in growth factor immobilization enabling the modulation of cellular response in naturally derived materials limits their usefulness for studying fetal membrane repair.

Here we employed a modular designed, biomimetic matrix to establish fetal membrane tissue models. This matrix can be rationally designed by modulation of cross-link density, incorporation of protease-sensitive sites, or modification with integrin ligands for cell adhesion [22][23]. As this matrix builds on a clean canvas and a limited number of functionally defined building blocks are employed, the effect of growth factors on cells can be studied independent of inherent environmental cues, present in naturally derived matrices such as collagen and fibrin. For the screening of potential chemotactic signals mesenchymal cells from amnion were chosen over cells from the chorion for several reasons: *i)* amnion is the mechanically stronger layer of the fetal membranes [48], and its ECM is mainly formed by the mesenchymal cells *ii)* the presence of fetal mesenchymal and epithelial cells in absence of maternal cells and vasculature allows for the harvest of a relatively pure primary cell population by a

simple isolation procedure *iii)* the simple tissue architecture of amnion can be recapitulated relatively easy *in vitro*.

As the microenvironmental signals required for fetal membrane healing are still largely unknown, the limited information derived from conventional 2D approaches [42][43][44][45] in combination with growth factors and cytokines identified as chemoattractants for mesenchymal cells from other than extraembryonic sources were taken as a starting point for our screening approach. We have shown that migration of hAMC can be stimulated in hydrogels as single cells or from tissue mimicking spheroids (microtissues) as well as from intact pieces of amnion tissue, none of which have been described before. hAMC could also be induced to proliferate in the 3D cultures but this response was always slower than migration. Significant differences in the amount of DNA and metabolic activity could only be seen after 10 days of culture in contrast to extremely fast (hours) migratory response to some factors.

Among the tested 10 growth factors and cytokines PDGF was the most potent factor for stimulating both migration and proliferation of hAMC, which was not surprising in the light of published literature on PDGF's effects on other mesenchymal cell types [25][49][50][51]. Also bFGF and EGF have been reported to have a role in maintaining mesenchymal cell self-renewal and acting as mitogens [52][53], whereas TGF-β has been associated with differentiation and tissue morphogenesis [54]. Interestingly cells stimulated with PDGF migrate predominantly by a mesenchymal-type mechanism whereas cells stimulated with bFGF, TGF-β and EGF exhibited a collective migration mode [32]. MMP inhibition was able to stop the migration of hAMC indicating that they are actively secreting MMPs which is necessary for remodelling the matrix into new tissue. As EGF in addition to its effect on hAMC can act on epithelial cells in the membrane, the combination of EGF and PDGF might be the most interesting for further investigations.

The strength and elasticity of fetal membranes is based on its collagen (primarily types I and III) and elastin content [55]. In order to repair membrane tissue, recruited amnion cells should retain their extracellular matrix production. In amnion, mesenchymal cells have been reported to be mostly responsible on the collagen production [56], thus only the expression in these cells were analysed. Although none of the migration or proliferation stimulating factors could enhance the production of ECM components prominently present in amnion, mRNA expression of cells cultured in the hydrogel system for up to four weeks remained at comparable levels to freshly isolated cells. As amnion cell adhesion to decellularized human amnion has been shown to be markedly improved when the membranes were stretched by clamping them on IVF plates [57] we suspect that mechanical loading might also influence the ECM production in hAMCs as observed in connective tissue cells [58]. Additionally, factors such as connective tissue growth factor (CTGF) which activate pathways leading to ECM production and fibroblast differentiation in mesenchymal cells [59] will be evaluated.

Our *in vitro* data demonstrate that different growth factors are capable of initiating cellular processes involved in healing. To avoid time consuming engineering and production of recombinant proteins, soluble growth factors were used for screening purposes. In order to inducing healing by biomimetic matrices in a therapeutically safe and sustainable manner growth factor release must be tightly controlled. Several growth factor immobilization strategies exist for the employed hydrogel system, which rely on molecular interactions with varying strengths and could be harnessed for the delivery of different recruitment, proliferation and extracellular matrix production inducing growth factors in a spatiotemporally controlled manner.

CONCLUSION

In conclusion, we have successfully used a fully synthetic ECM analog for identifying novel growth factors stimulating primary amnion mesenchymal cells to undergo processes necessary for reconstructing injured tissue. This knowledge can serve as a stepping stone to next generation biomaterials for studying fetal membrane repair and creating treatment options for iPPROM by modulating the healing response.

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Supplementary information

Materials and methods

Cell isolation

Briefly, amnion was separated from chorion and washed several times with phosphate buffered saline (PBS) with 1% penicillin-streptomycin (P/S) (Gibco, Grand Island, NY, USA). Amnion was digested with 0.25% Trypsin (Gibco) for 5 min at 37 °C and the resulting supernatant was discarded. Amnion was then washed two times and digested with 1.2 U/ml Dispase II (Roche, Basel, Switzerland) in PBS for 60 min. Subsequently the epithelium was scraped off with a cell scraper and epithelial cells (termed hAEC) taken in culture. The remaining stromal part of the membrane was cut into small pieces and digested with 2 mg/ml Collagenase A (Roche) in PBS for two hours. Mesenchymal cells (termed hAMC) obtained from this digestion as well as the hAEC were cultured in alpha minimum essential medium (αMEM, Gibco) with 10% fetal bovine serum (FBS, Gibco) and 1% P/S and grown at 37 °C and 5% CO_2 in a humidified atmosphere. Cells were used for the experiments between passages 0 to 2.

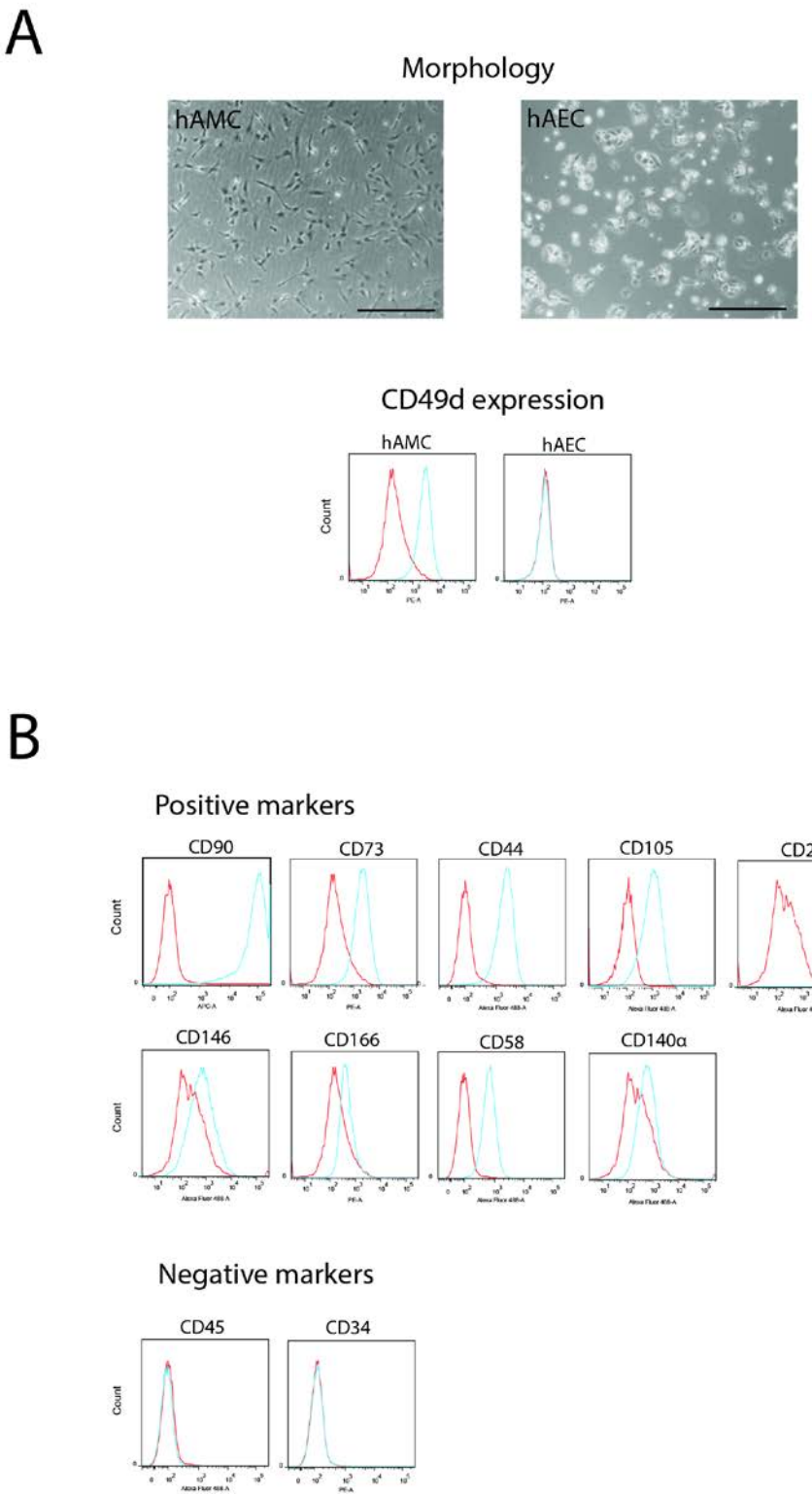
Characterization by FACS

Immunophenotypic analyses were performed on amnion mesenchymal cells (hAMC) from three isolations. Cells were collected, washed, resuspended in 100 µl PBS with 1mM EDTA and stained with mouse monoclonal anti-CD29 (Biolegend, San Diego, CA, USA), mouse monoclonal anti-CD34-PE (Miltenyi Biotec, Bergisch-Gladbach, Germany), mouse monoclonal anti-CD45-FITC (Miltenyi Biotec), mouse monoclonal anti-CD44-FITC (BD Pharmingen, Palo Alto, CA, USA), mouse monoclonal anti-CD73-PE (BD Pharmingen), mouse monoclonal anti-CD90-APC (BD Pharmingen), mouse monoclonal anti-CD105 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-CD140α (Biolegend), mouse monoclonal anti-CD166-PE (BD Pharmingen) or mouse monoclonal anti-CD146 (Chemicon) antibodies for 30 min. Samples which were not directly incubated with fluorochrome conjugated antibodies were washed and stained with DyLight™ 488 Goat anti-mouse IgG (Biolegend) for 30 min. After staining, cells were washed and fixed. Samples were analyzed using the BD FACS-Canto II and DIVA software, recording at least 10 000 events per marker. Comparative analysis was performed with FlowJo Version 7.6.4 (Tree Star, Inc., Ashland, OR, USA). CD49d has been used as a marker to distinguish between amnion epithelial and mesenchymal cells [20], so its expression was analysed by flow cytometry in hAMC and hAEC to verify the purity of the populations using mouse monoclonal anti-CD49d-PE (Biolegend). Appropriate isotype controls from manufacturers were used as negative controls.

Results

Characterization of human amnion mesenchymal cells (hAMCs)

Cell isolations from amnion samples consistently resulted in separate fractions of mesenchymal and epithelial cells based on both cell morphology (Supplementary Figure 1A) and expression of CD49d (Supplementary Figure 3.1A), which has been reported to be expressed only by the mesenchymal cells in amnion [20]. Amnion mesenchymal cells were also positive for CD44, CD73, CD90 and CD105 from the standard mesenchymal markers and negative for CD45 and CD34 excluding hematopoietic and endothelial contamination (Supplementary Figure 1B). They also expressed CD29, CD140α, CD146 and CD166 (Supplementary Figure 3.1B) associated with mesenchymal stem cells from various tissues.



Supplementary figure 3.1 Characterization of hAMC and hAEC. (A) Representative bright field images of isolated hAMC and hAEC on tissue culture plastic and FACS analysis of CD49d expression confirming the purity of the populations. Scale bars, 500 μm. (B) FACS analysis of

hAMCS stained against characteristic mesenchymal (CD90, CD73, CD44, CD105, CD29, CD146, CD166, CD58 and CD140α) endothelial (CD34) and hematopoietic markers (CD45 and CD34).

Supplementary video legends (videos available online):

Microtissues consisting of hAMCs were embedded in PEG-based biomimetic hydrogels containing 50 μM RGD and stimulated by the addition of growth factors to the cell culture medium. Migration was visualized using time lapse microscopy (images captured every 10 minutes for 24 hours). Scale bars, 100 μm.

S1 Migration time course in response to no growth factor during the first 24 hours.

S2 Migration time course in response to 100 ng/ml PDGF during the first 24 hours.

S3 Migration time course in response to 100 ng/ml bFGF during the first 24 hours.

S4 Migration time course in response to 100 ng/ml EGF during the first 24 hours.

S5 Migration time course in response to 100 ng/ml TGF-β during the first 24 hours.

CHAPTER 4 Human amniotic fluid cells for engineering vascularized fetal bone grafts

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ABSTRACT

Cleft palate is one of the most common birth defects for which current treatment options rely on multiple surgical interventions. Since in young children adequate autologous bone is very limited, harvesting bone results in significant donor site morbidity, and the current non-living grafting materials do not comply with the growth of the craniofacial structures, the use of tissue engineered grafts has been suggested.

Such engineered tissues would offer the advantage of becoming functionally integrated and undergoing adequate remodeling during tissue growth. However, the use of tissue engineered constructs is currently limited by the lack of i) appropriate autologous cell sources and ii) structural organization and vascularization. Fetal tissue engineering using autologous amniocentesis-derived and ethically unproblematic amniotic fluid cells (AFCs) has been envisioned but *in vivo* results have been moderate due to lacking vascular supply in the middle of the graft. Consequently, we propose the formation of next generation three dimensionally (3D) structured and vascularized bone tissue replacements, engineered from patients own, phenotypically characterized and differentiated stem cells.

Fully synthetic extracellular matrix mimicking poly(ethylene glycol) (PEG)-based hydrogels were first used to evaluate 3D osteogenic differentiation of AFCs and creation of vascular structures from AFC derived endothelial cells (enAFC) and undifferentiated AFC either in random or organized channel cocultures *in vitro* and *in vivo*. In the future these approaches can be combined in an osteogenic matrix with a channel perfused with enAFC for treating congenital bone defects.

Keywords: amniotic fluid cells, poly(ethylene glycol), hydrogel, bone regeneration, pre-vascularization, fetal tissue engineering

INTRODUCTION

Cleft palate is one of the most common birth defects occurring in 1 in 300 to 3000 live births depending on racial distribution and the concurrence of environmental factors [1]. Current treatment options rely on multiple soft and hard tissue surgical intervention presenting a significant burden to the affected individuals as well as to the socioeconomic system. Operative interventions attempt to establish the structural and functional integrity of the affected tissues right after birth or when repair is feasible in terms of health and developmental status of the child. In young children adequate autologous bone is even more limited than in adults, harvesting bone results in significant donor site morbidity, and non-living grafting materials, such as hydroxyapatite, Gore-Tex®, Teflon®, or Dacron® commonly used to repair diaphragm defects, to substitute heart valves, or to bridge cleft palates [2-4], do not comply with the growth of the child. Thus, tissue engineering has been envisioned for the treatment of cleft palate (reviewed in [5]).

Such engineered tissues would offer the advantage of becoming functionally integrated, undergoing remodeling during tissue growth, and could thus limit the number of surgical interventions needed. However, the use of tissue engineered constructs is currently limited by the lack of i) appropriate autologous cell sources and ii) structural organization and vascularization. Expansion of mesenchymal stem cells from bone marrow or fat of newborns to sufficient cell numbers is not feasible. Thus, for fetal tissue engineering, the use of autologous amniocentesis-derived and thus ethically unproblematic fetal amniotic stem cells (AFSCs) has recently been envisioned. The successful construction of functional bone tissue grafts would represent a breakthrough for cleft palate treatment.

It has been established that amniotic fluid contains stem cells and differentiated cells derived from all three germ layers [6]. These cells are shed from the developing fetus or from the fetal membranes and in culture give rise to epithelial-like, amniotic fluid-specific and fibroblast-like cells. Genome wide analysis of amniotic fluid cells has shown that they to have a unique stem cell signature and possess characteristics related to pluripotency [7]. Correspondingly their conversion to induced pluripotent stem cells (iPS) has also been shown to be much easier than of adult cells [8]. In contrast to embryonic stem cells or iPS cells AFSCs had reduced plasticity and were non-tumorigenic [9]. Many investigators have demonstrated the multilineage differentiation capabilities of AFSCs [9, 10], among them conversion to osteoblasts [11] and endothelial cells [12]. The large proliferative potential (>250 population doublings) of clonally expanded AFSCs with mesenchymal properties has also been demonstrated as well as conservation of telomerase activity up to late passages [9].

For pediatric applications AFSCs have been considered as alternatives, which would allow the production of tissues in parallel to the ongoing pregnancy and the use of autologous material alone. In comparison to bone marrow mesenchymal stem cells, AFSCs have shown to be superior for bone and vasculature tissue engineering, since in addition to being able give rise to endothelial and osteoblastic

cells, they can secrete various soluble factors enhancing angiogenesis and healing [13]. In a rabbit preclinical model AFSCs have been employed for the repair of defective fetal bone tissue [14]. However, in this as well as in other studies, large non-vascularized constructs will mostly undergo apoptosis and thus result in limited functional integration.

Vascularization is needed for any tissue engineered constructs exciding the diffusion limits of nutrients and oxygen in size. Core of a construct larger than this will become hypoxic and *in vivo* attract the host vasculature to invade. However, this process leading to a fully vascularized implant takes weeks even for a millimeter-sized construct during which time the inner parts will become necrotic compromising the overall viability and integration [15]. The concept of pre-vascularization encompasses that vascular networks engineered *in vitro* will provide established conduits that can be rapidly perfused with blood after *in vivo* implantation. Pre-vascularization strategies for tissue engineered constructs include co-cultures of endothelial and mesenchymal cells from various sources [16] and engineering approaches such as scaffold structures imitating blood vessels [17].

Modular designed biomimetic materials which are chemically, physically and biologically defined have been developed, starting from biologically inactive building blocks. The previously developed TG-PEG platform combines some of the properties of the naturally derived matrices, such as factor XIII-mediated (FXIII) cross-linking scheme and growth factor immobilization with the properties of star-shaped poly(ethylene) glycol (PEG)-based materials [18]. These biomimetic matrices are formed under near to physiological conditions upon addition of FXIIIa and allow the encapsulation of cells and the specific covalent immobilization of growth factors, without compromising their activity, as has been demonstrated using engineered VEGF [19] and PDGF [20]. Furthermore, such matrices can be engineered regarding presentation of prototypic integrin ligand RGD, susceptibility towards proteolytic degradation, and continuous or triggered release of growth factors and they have been used to study *in vitro* cell migration [21, 22] and *in vivo* healing behaviour [22].

To facilitate the early ingrowth of vascular structures, we created perfusable channels within a cell containing hydrogel matrix and compared this approach to self-organizing cocultures. We also initiated combining self-organizing capillary structures from random cocultures in the bulk of the construct with an organized endothelialized channel to benefit from both approaches. Finally we combined the pre-vascularization process with osteogenic differentiation.

Differentiation of amniotic fluid cells to bone in three dimensional scaffolds to repair bone defects has been reported previously [11, 23, 24]. Amniotic fluid cells were also previously used to create capillary networks in vitro using fibrin/PEG hydrogels [25]. We hypothesize that AFCs can be differentiated into osteoblasts and endothelial cells and that their spatial 3D arrangement in mechanically and biochemically defined microenvironments will be sufficient to create bone-like constructs which consists of autologous cells, are vascularized and thus readily integrate to the host and can be transplanted immediately after birth. We propose the formation of next generation three

dimensionally (3D) structured and vascularized bone tissue replacements, engineered from patients own, phenotypically characterized and differentiated stem cells.

MATERIALS AND METHODS

Cell isolation, culture and characterization

Amniotic fluid was acquired from sampling for diagnostic purposes (chromosomal abnormalities) between 16 to 18 weeks of gestation. Patients were informed (written and orally) that amniotic fluid cells would be used for research purposes as described in this article (approved by the Ethics Committee StV23/2006), and written informed consent was obtained from the participants. After ultrasound assessment of fetal biometry and amniotic fluid index, ultrasound guided abdominal amniocentesis was performed under sterile conditions using a 22 G needle. Sample of 3 ml was diluted in 27 ml of expansion medium (MEMα with 15% Embryonic stem cell qualified fetal bovine serum (ES-FBS) and 1% penicillin-streptomycin (P/S), all from GIBCO) and plated on three 10 cm cell culture dishes. Rapidly growing colonies of small cells with mesenchymal morphology were selected for further expansion. Cells were characterized in terms of their phenotype and self-renewal and differentiation potential (supplementary materials and methods).

For culture in the hydrogels, optimized 3D expansion media composed of 1:4 mixture of EGM-2 medium and MEMα with total 10% of ES-FBS and *50 μg/ml ascorbic acid* and additional 5 ng/ml basic fibroblast growth factor (bFGF, Peprotech) and 25 ng/ml of platelet derived growth factor (PDGF-BB, Peprotech) was used.

Endothelial and osteogenic differentiation

Endothelial differentiation was initiated by culturing cells on flasks coated with rat tail collagen I (SIGMA) in EGM-2 (Lonza) medium with 10% ES-FBS with additional 50 ng/ml of VEGF-165 (Peprotech, Rocky Hill, NJ, USA) added in the medium. Medium was changed every third day. Endothelial differentiation was evaluated based on cell morphology and immunostainings (Supplementary information).

Osteogenic differentiation on plastic is described in supplementary materials. For osteogenic differentiation of hydrogel encapsulated cells, cells were first cultured for 5 days in expansion media in order to allow spreading and production of fibronectin and collagen I which improved the viability upon osteogenic stimulation (data not shown). Osteogenic media was composed of DMEM with 10% ES-FBS, 1% P/S, 50 μg/ml ascorbic acid, 10mM beta-glycerol phosphate and 40 ng/ml (102.4 nM) Dexamethasone (all from Sigma-Aldrich, Switzerland) or 100 ng/ml human recombinant bone morphogenic protein-2 (BMP-2, produced as described previously [26]). Medium was changed every third day.

Osteogenic differentiation was evaluated by colorimetric alkaline phosphatase (ALP) staining and alizarin red s staining for calcium deposition. ALP staining was performed using SIGMA*FAST* BCIP/NBT tablets (Sigma-Aldrich) which were solubilized in double distilled sterile water. Medium was removed from the hydrogels, they were washed once with PBS and covered with the staining solution for 20 minutes after which it was replaced with PBS. For Alizarin red S staining samples were fixed with 4% paraformaldehyde for 20 min, washed once with water and stained with 2% Alizarin red S in ddH_2O (pH 4.1) for 5 min and washed extensively with water. Samples were imaged with a digital camera and with a light microscope (Carl Zeiss AG, Jena, Germany).

PEG hydrogel formation

TG-PEG hydrogel formation was performed as previously described [18, 19]. Briefly, functionalization of eight-arm PEG-vinylsulfone (PEG-VS) with the FXIII substrate peptides glutamine acceptor substrate or lysine donor substrate containing a MMP-sensitive linker resulted in n-PEG-Gln or n-PEG-$MMP_{sensitive}$-Lys monomers. Covalently cross-linked hydrogels were formed by the addition of 10 U ml^{-1} of thrombin-activated factor XIIIa to a tris buffered saline (50 mM, pH 7.6) solution containing besides a stoichiometrically balanced amount of n-PEG- $MMP_{sensitive}$Lys and n-PEG-Gln 50 mM calcium chloride and 100 µM Gln-RGD.

Cell encapsulation into hydrogels

Suspension containing 7 x 10^6 cells mL^{-1} in culture medium was added to the gel mass right before FXIIIa. After the addition of FXIIIa gels were either formed as 20 or 50 µL disks between two hydrophobic glass slides separated by spacers (1.25 or 2 mm) or cast in PDMS moulds for channels constructs (design of the mold described in [27]). In these constructs a 50 µL gel was formed around a PLL-g-PEG coated tungsten rods (Ø 500 µm) which are non-adhesive to TG-PEG [28] and could be removed from the mold at a later time point while perfusing the remaining empty channel with cells or culture media. Gelation of the hydrogels occurred within few minutes at room temperature, but the cross-linking reaction was allowed to further proceed for 30 min at 37 °C in a humidified incubator.

Coculture experiments

After endothelial differentiation of the AFC (supplementary information and supplementary Fig. 3B) cocultures of undifferentiated AFC were formed either by encapsulating cells and letting them organize themselves (4:1 enAFC/AFC, called random cocultures from here on) or by perfusing the enAFC in the preformed channel within an AFC or enAFC+AFC containing hydrogel (called structured cocultures from here on). Channels were perfused by dispensing 10 µL of cell suspension (2 x 10^7 cells/ml) into a well connected with the channel forming rod which was then slowly pulled out of the mold resulting in the cell suspension being aspirated into the channel.

Analysis of cell viability

Cell viability in 3D hydrogel cultures after one week was examined by LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR, USA). Briefly, ethidium homodimer-1 and calcein AM from the kit were diluted in 1:1000 ratio in culture medium and gels were stained with this solution for 10 min in cell culture incubator before imaging.

Immunocytochemistry and microscopy

Samples were fixed with 4% paraformaldehyde for 20 min followed by 5 min incubation in 0.1 M glycine and two washes with PBS after which they were permeabilized and blocked with 1% BSA and 0.2% Triton X-100 in PBS for 60 min before staining. For collagen I staining samples were incubated with the primary antibody prior to fixation for 60 min in the cell culture incubator and fixed afterwards. F-actin was stained with Phalloidin-Alexa fluor 546 (Invitrogen, Carlsbad, CA, USA) in PBS with 1% BSA (Bovine serum albumin) overnight at 4˚C. For immunostainings, following primary antibodies were used at 1:100: mouse monoclonal anti-collagen I (Abcam, Cambridge, MA, USA) and FITC-conjugated sheep polyclonal anti-fibronectin (Abcam). DyLight™ 488 Goat anti-mouse IgG (Biolegend) was used as secondary antibodies at 1:200 when needed. After washing the samples three times in PBS, cell nuclei were stained with 5 ng/mL DAPI (40,6-diamidino-2-phenylindole) (Sigma Aldrich) in PBS for 10 min at room temperature. Sample evaluation was performed by either confocal laser scanning microscopy (Leica TCS SP5) or epifluorescence microscope (BM550B, Leica Microsystems, Germany).

In vivo experiments

In vivo experiments were approved by the local veterinarian department (permit number 141/2012) and conducted in accordance with the Swiss law of animal protection. All mice used in this study were female NMRI nude mice (HsdCpb:NMRI-Foxn1^{nu}) purchased from Harlan and 5-7 weeks old at the start of the experiments.

Functionality of *in vitro* established vascular structures was evaluated *in vivo* by implanting them subcutaneously on each side lateral of the dorsal midline region on the backs of mice (four implants per mice in randomized order). Bone formation in pre-differentiated osteogenic constructs with or without pre-vascularization was assessed in the same model. Animals were sacrificed after at predefined time points post-operation for analysis by micro-CT and histology.

Histology

Collected samples were fixed with either 4% formalin for 4 hours and embedded in paraffin for sectioning. For histological analysis of samples containing mature bone, samples were decalcified using USEDECALC (MEDITE, cat. no. 40-3310-00). Four µm sections were made though the embedded samples and stained with hematoxylin-eosin (HE). Images were acquired using the KS400 image

analysis system (Carl Zeiss AG, Jena, Germany) linked to a Zeiss Axioskop microscope 50 equipped with a digital camera (Axio Cam MRc5, Carl Zeiss AG, Jena, Germany).

Micro computed tomography

After fixation in formalin and storage in PBS, subcutaneous implants were scanned in a micro-CT 40 (Scanco Medical AG) operated at energy of 55 kVp and intensity of 72 μA. Scans were executed at a high-resolution mode resulting in a voxel size of 10 μm. In reconstructed images bone tissue was segmented from background using a global threshold of 35.3 % of maximum grey value. 3D surface rendered images were created using the ImageJ 3D viewer plugin.

RESULTS

Cell viability and morphology in and on top of hydrogels

As a starting point for combining the TG-PEG hydrogel with amniotic fluid cells, viability, spreading and ECM production of undifferentiated AFCs encapsulated in the TG-PEG hydrogel was evaluated. Preliminary experiments indicated that the expansion media used for 2D culture was not sufficient in supporting the cells in hydrogel cultures, so 3D expansion media was developed (data not shown). After one week of culture in TG-PEG hydrogels AFCs had spread and formed interconnected networks spanning throughout the gel when cultured in 3D expansion media whereas the normal 2D media resulted in round cell morphology (Fig. 4.1A). Live-dead staining also confirmed that most of the cells in this media had remained viable and stained green in comparison to the 2D expansion media in which most cells were dead (Fig. 4.1A). Endothelial differentiated AFC (enAFC, characterization in supplementary information) in 3D expansion media adhered on top of a TG-PEG hydrogel and formed a confluent monolayer (Fig. 4.1B) which was taken as a confirmation that they could adhere onto the channel lumen as well.

Time series (1-14 days) of fibronectin and collagen I deposition showed that both ECM components were produced by AFC as early as one day after encapsulation into the synthetic matrix (Supplementary fig. 4.3). Immunostaining for fibronectin reached its peak level at day 7 after which it appeared to stain rather constant (Fig. 4.1C) whereas collagen I displayed the strongest staining later at day 11 (Fig. 4.1C).

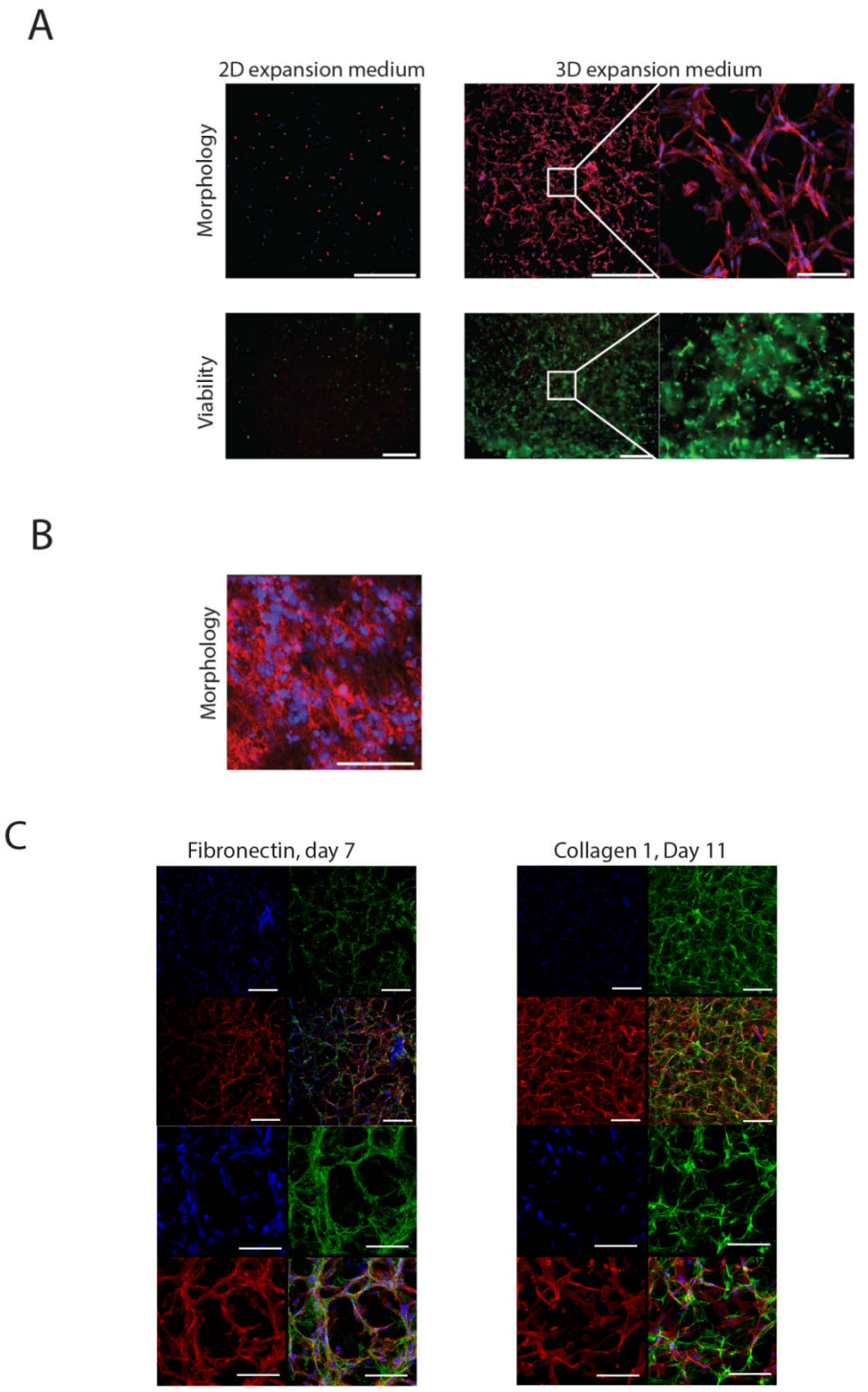

Figure 4.1 AFC morphology, viability and extracellular matrix production in TG-PEG hydrogels. (A) 6.5x104 AFCs/ml were encapsulated in biomimetic TG-PEG hydrogels containing 100 μM RGD

and cultured either in 2D expansion medium (MEMα with 10% of ES-FBS) or in 3D expansion medium (1:4 mixture of EGM-2 and MEMα with 10% of ES-FBS, 50 ug/ml ascorbic acid, 5 ng/ml bFGF and 25 ng/ml PDGF). Cell morphology and viability in hydrogels after seven days of culture displaying either round cells and mostly dead cells (2D expansion medium) or spreading and network formation of viable cells (3D expansion medium). Red Phalloidin-Alexa fluor 546 and blue DAPI. Scale bars, 200 µm (low magnification) and 100 µm (high magnification). (B) Monolayer of enAFC on top of a TG-PEG hydrogel after seven days of culture. Scale bar 100 µm. (C) Immunostaining for fibronectin at day 7 and collagen 1 at day 11. Green fibronectin or collagen I, red Phalloidin-Alexa fluor 546 and blue DAPI. Scale bars, 200 µm (low magnification) and 100 µm (high magnification).

Osteogenic differentiation of amniotic fluid cells in TG-PEG hydrogels

Osteogenic differentiation *in vitro* in TG-PEG hydrogels was evaluated by alkaline phosphatase (ALP) activity and alizarin red S staining after 3, 7, 18, 23 and 28 days. Encapsulated cells were either cultured in 3D expansion medium that contained no or 100 ng/ml BMP-2, or in osteogenic medium that contained 40 ng/ml dexamethasone or 100 ng/ml BMP-2.

ALP staining was present in all of the samples after 3 days of differentiation (Fig. 4.2A). For samples that were grown in expansion medium with or without BMP the activity remained unchanged over the course of the experiment. Whereas, gels that were incubated in osteogenic media containing dexamethasone or BMP-2 appeared white and the ALP staining became less apparent after 7 days of culture.

Differences in alizarin red S staining between the control gels kept in the 3D expansion media and the gels in the osteogenic media with dexamethasone or BMP-2 were obvious after 7 days (Fig. 4.2B) and became continuously more pronounced with increasing time of differentiation. 13 days was deemed sufficient time for pre-differentiation for *in vivo* experiments as mineralization was evident (Fig. 4.2C). Additionally, when gels were incubated in expansion medium their dimension got significantly reduced due to cells overgrowing the original gel, consistent with ongoing proliferation. In contrast, gels in osteogenic conditions largely retained the original dimensions due to differentiation and reduced proliferation of cells.

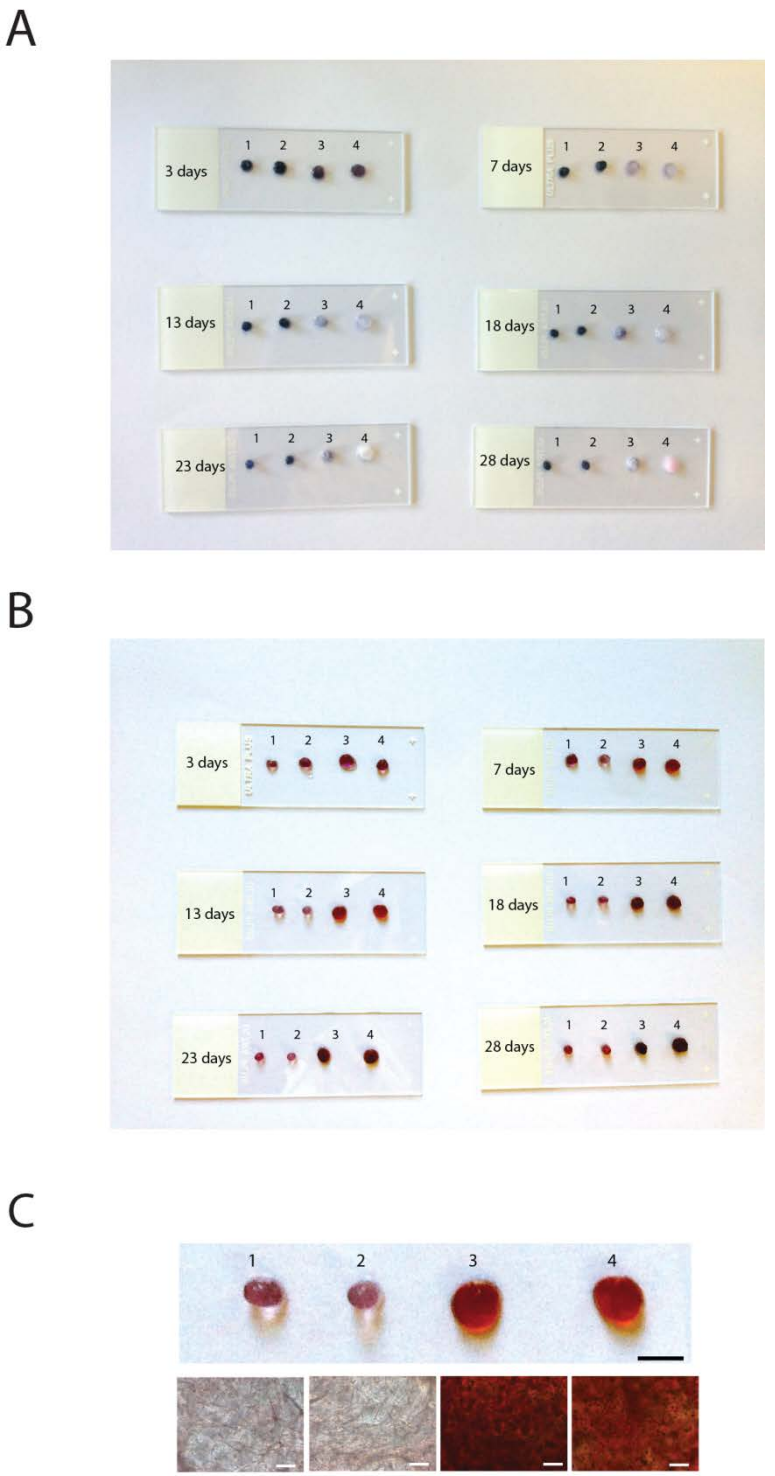

Figure 4.2 In vitro osteogenic differentiation of AFC in TG-PEG hydrogels. AFC encapsulated in TG-PEG hydrogels were expanded for 5 days prior to osteogenic differentiation in the following conditions: 1. 3D expansion media 2. 3D exp. media + 100 ng/ml BMP-2 3. Osteogenic media with 100 ng/ml BPM-2. (A) Colorimetric alkaline phosphatase staining after 3, 7, 13, 18, 23 and 28 days in different osteogenic and control conditions. (B) Alizarin red S staining after 3, 7, 13, 18, 23 and 28 days in different osteogenic and control conditions. (C) Bright field images of alizarin

red S staining after 13 days in different osteogenic and control conditions. Scale bars, 4 mm and 200 μm.

Ectopic bone formation of *in vitro* differentiated constructs

In order test the *in vivo* bone forming capacity of pre-differentiated AFC-based tissue constructs, AFCs were expanded for 5 days in TG-PEG hydrogels, differentiated with osteogenic media containing dexamethasone for 14 days and subsequently transplantation in subcutaneous pouches of immunodeficient mice. After 4 and 8 weeks micro-CT inspection of the explanted tissue constructs displayed mineralization the pre-differentiated samples whereas no signal could be detected in control samples (Fig. 4.3A). Whereas hematoxylin-eosin stained histological sections did not yet show any structures typical to mature bone after 4 weeks, after 8 weeks some bone like structures had started forming especially in the edges of the implants (Fig. 4.3B). Pre-differentiated samples displayed condensed networks of cells with long extensions whereas control samples seemed to contain much less cells and more intact gel. This might indicate that most of the undifferentiated cells migrated out of the constructs which originally they had extremely high cell densities. Interestingly pre-differentiated samples also had perfused vascular structures spanning throughout the osteogenic matrix which were absent from the control samples (Fig. 4.3B).

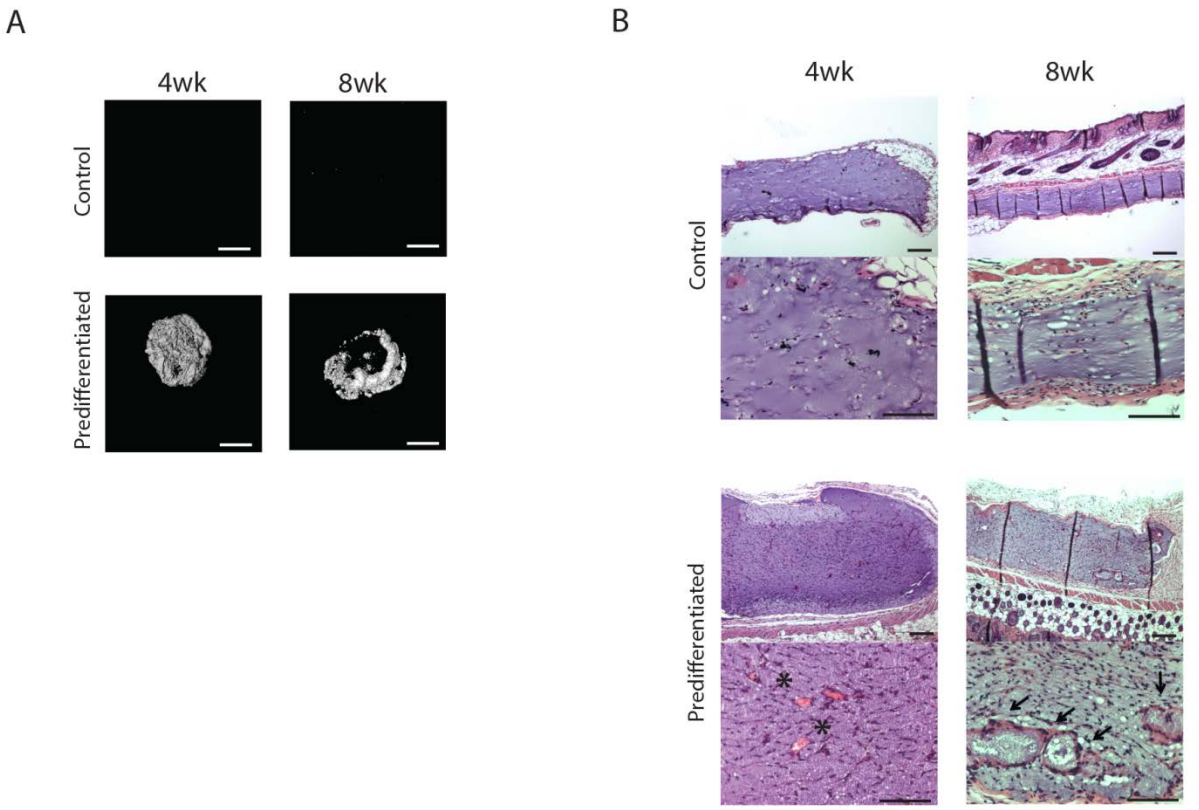

Figure 4.3 In vivo evaluation of pre-differentiated osteogenic constructs. AFC encapsulated in TG-PEG hydrogels were expanded for 5 days and differentiated in osteogenic media with dexamethasone for 14 days (control samples kept in 3D expansion media) after which they were implanted subcutaneously in the backs of nude mice (A) Micro-CT evaluation of bone formation after 4 and 8 weeks in vivo. Scale bars 1mm. (B) Hematoxylin-Eosin staining of paraffin embedded decalcified tissue sections after 4 and 8 weeks in vivo. Perfused vascular structures are indicated with an asterisk and bone like structures with arrows. Scale bars, 200 μm (low magnification) and 100 μm (high magnification).

Formation of vascular structures in hydrogel cocultures of AFC and enAFC *in vitro*

Grafted tissues often suffer from low engraftment largely due to insufficient early integration with the host vasculature which could be partially overcome by pre-vascularization. Therefore tissue engineered constructs were formed by relying on self-organization of encapsulated cells or the perfusion of endothelial cells into preformed channels. As we aimed for the generation of fetal tissues, AFCs were differentiated for 10 days by an earlier published protocol toward endothelial cells [12]. Endothelial differentiated amniotic fluid cells (enAFCs) cells resumed an endothelial morphology and expressed the typical endothelial markers VEGFR2, CD31, Dll4, Notch 1 and vWf. As confirmed by immunohistochemistry (Supplementary Fig. 4.2B). enAFCs adhered on top of a TG-PEG hydrogel and

formed a confluent monolayer (Fig. 4.1B) which was taken as a confirmation that they could adhere onto the channel lumen as well.

After endothelial differentiation of the AFC (supplementary information and supplementary Fig. 4.3B) cocultures of undifferentiated AFC were formed either by encapsulating cells and letting them organize themselves (called random cocultures from here on) or by perfusing the enAFC in a preformed channel within an AFC containing hydrogel. Cells in random cocultures networks displayed early lumen formation after 2 days in culture (Fig. 4.4A). enAFC perfused into the channel lined within 24 hours and formed a stable channel (Fig. 4.4A).

Integration of pre-vascularized constructs *in vivo*

To determine the influence of structured or randomly arranged vascular networks for the integration into the host vasculature and therefore likely improved engraftment efficiency of engineered tissues, undifferentiated AFCs were encapsulated in TG-PEG hydrogels *i)* as random cocultures with enAFCs (in a ratio 4 :1) *ii)* in presence of an empty channel *iii)* in presence of enAFC seeded channel *iv)* as random coculture (in a ratio 4 :1) containing an empty channel and *v)* as random cocultures (in a ratio 4 :1) containing an additionally enAFC seeded channel. Constructs containing in total $6.5x10^4$ cells/ml of hydrogel were allowed to maturate one week in culture before their subcutaneous implantation in immunocompromised mice for 2 weeks. Histological evaluation of explanted tissue constructs showed that random cocultures alone or around the channel resulted in perfused capillaries of 5-10 µm (Fig. 4.4B). The channel with enAFC facilitated the ingrowth of host connective tissue and condensed arrangement of perfused larger vascular structures of 15-30 µm (Fig. 4.4B). Constructs with only AFC did not have vascular structures within them (Fig. 4.4B).

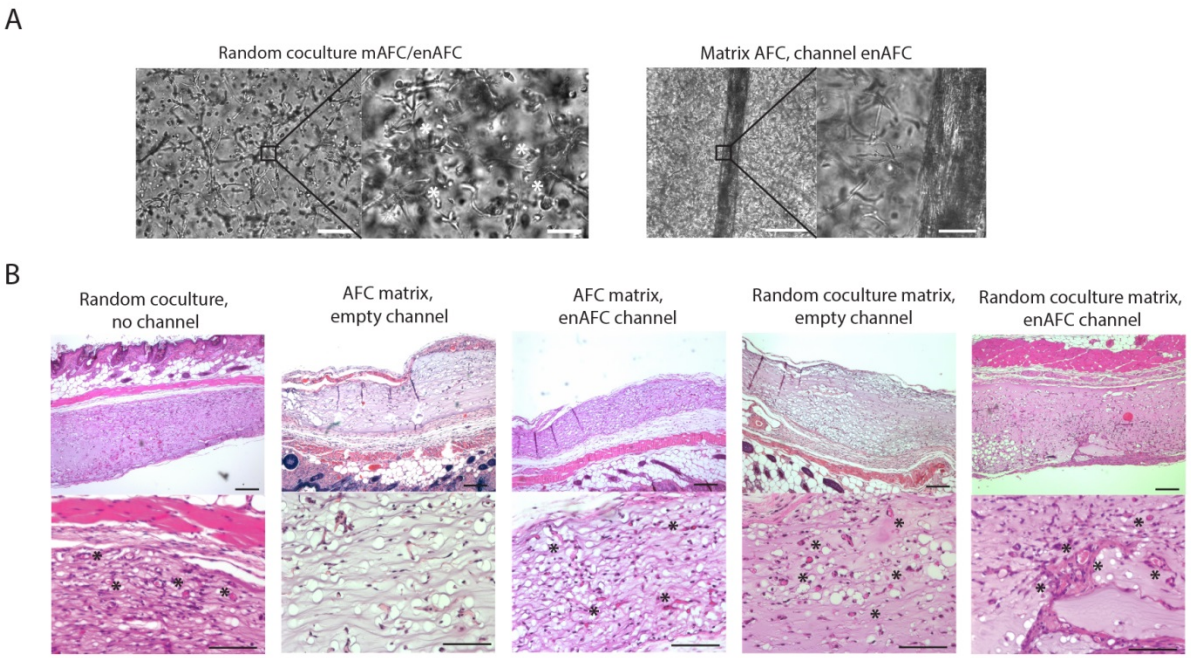

Figure 4.4 Random and organized cocultures of AFC and enAFC in vitro and in vivo. Cocultures formed either by mixing 4:1 AFC:enAFC (total 6.5x104 cellss/ml) or perfusing enAFC in a channel within AFC matrix. (A) Cocultures after 5 days of in vitro culture in 3D expansion medium. Random cocultures displaying lumen formation (*) and enAFC lining the channel. (B) Hematoxylin-eosin staining of pre-vascularized constructs after 2 weeks of subcutaneous implantation. Scale bars, 200 μm (low magnification) and 100 μm (high magnification).

Ectopic bone formation of *in vitro* differentiated and pre-vascularized bone construct

Since both the presence of endothelial cells and preformed channels inside tissue engineered constructs led to improved vascularization, we next aimed evaluating whether these pre-vascularization strategies could lead to improved bone tissue engraftment. Therefore AFC containing cellular constructs which contained or did not contain channels were formed in TG-PEG hydrogels and expanded for 5 days. After differentiation for 14 days in osteogenic media which contained no or 40 ng/ml dexamethasone, in some samples enAFC were perfused into the channel and allowed to maturate for one day before implantation into subcutaneous pouches of mice. Micro-CT inspection of the explanted constructs after 8 weeks displayed mineralization in the pre-differentiated samples with or without pre-vascularization, whereas no signal could be detected in control samples (Fig. 4.5A). Pre-vascularized samples were mineralized everywhere compared to the non-pre-vascularized samples which exhibited more mineral deposition on the edges of the constructs (Fig. 4.5A). Histological evaluation by hematoxylin-eosin stained paraffin sections displayed bone like structures in both pre-differentiated groups. However, the samples with pre-vascularization bone formation was more pronounced and taking place throughout the hydrogel (Fig. 4.5B).

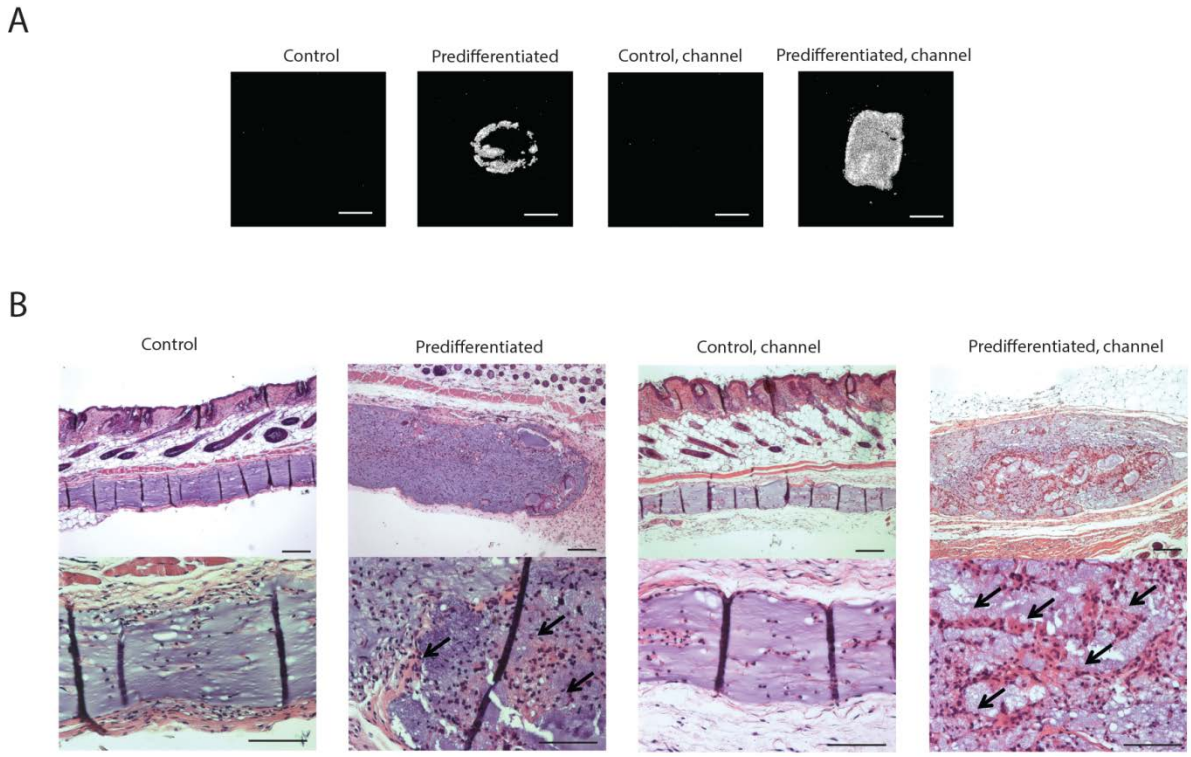

Figure 4.5 Ectopic bone formation and in pre-vascularized constructs. AFC encapsulated in TG-PEG hydrogels with or without channels were expanded for 5 days and differentiated in osteogenic media with dexamethasone for 14 days after which the channels were perfused with enAFC and cultured for 24 hours. Constructs were then implanted subcutaneously in the backs of nude mice (A) Micro-CT evaluation of bone formation after 8 weeks *in vivo*. Scale bars 1mm. (B) Hematoxylin-Eosin staining of paraffin embedded decalcified tissue sections after 8 weeks *in vivo*. Bone like structures are indicated with arrows. Scale bars, 200 µm (low magnification) and 100 µm (high magnification).

DISCUSSION

We describe the formation of pre-vascularized bone tissue engineered constructs derived from fetal derived human AFCs, which can be harvested at the second trimester of gestation by routine amniocenthesis procedure. Tissue engineering and cell-based therapies during the perinatal period have remained underexplored even though major congenital anomalies are present in approximately 2.4% of all newborns [29]. The use of amniotic fluid-derived cells (AFCs) has attracted increasing attention in recent years since at least some of these cells seem to be in a rather undifferentiated state and could give rise to cells with a broad spectrum of differentiation [9, 10].

In this work we explore this potential by the specific differentiation towards both osteogenic and endothelial lineages. In line with previous reports we observed osteogenic differentiation by dexamethasone [30] and endothelial differentiation by culture in endothelial growth medium with additional VEGF-165 [12]. We could also show osteogenic differentiation with BMP-2 which has not been published before, though BMP-7 has been used to differentiate AFCs [31]. Since the herein used

AFCs are derived from pooled colonies it remains unclear whether individual clones with higher proliferation and differentiation potential would exist, which would make this process more efficient. Recent literature indicates that indeed large variations in phenotype and function of such cell clones might exist [32] there are currently no definitive markers that would allow to select for better suitable clones.

Using modularly designed synthetic biomimetic hydrogels it is possible to carefully control biochemical signals and mechanical stiffness and thus study cellular response to isolated biological parameters. Surprisingly, such hydrogels comprising the commonly used integrin ligand RDG were not supporting the survival and spreading of AFCs in 3C culture when normal 2D expansion medium was used. Therefore different integrin ligands and cell culture medium additives were evaluated (data not shown) in order to obtain optimized 3D culture conditions.

Here we have employed such matrices first to evaluate 3D osteogenic differentiation of AFCs and then for creation of vascular structures from AFC derived endothelial cells (enAFC) and undifferentiated AFC either in random or organized channel cocultures *in vitro* and *in vivo*. Our data indicates that both the pre-vascularization by allowing self-organization of undifferentiated AFCs and enAFCs and the introduction of channel into the hydrogel lead to an improved vascularization of a tissue graft. While the presence of enAFCs within the bulk of the AFC seeded hydrogel likely results in a more efficient integration with the host vasculature, the existence of a large channel could additionally improve the penetration of the host tissue. The improved formation of bone tissues by pre-differentiated and pre-vascularized tissue graft indeed indicates that the integration with host tissue is improved, resulting in better survival of transplanted cells. However, experiments showing the engraftment, survival, localization and function of transplanted human endothelial or bone cells are still ongoing. Additionally experiments combining these two approaches in an osteogenic matrix with a channel perfused with enAFC for treating congenital bone defects are currently ongoing and discussed in chapter 5 of this thesis.

Cells encapsulated in a synthetic hydrogel would ideally adhere to provided adhesion sites and begin spreading and degrading the hydrogel and replace the synthetic material with their own extracellular matrix. This switch between the original material and new ECM should be tuned so that construct remains mechanically stable at all times and the composition of the forming ECM regulated with cues in the hydrogel or culture medium. Collagen I is the most abundant ECM protein in the human and it also represents 85-90% of total organic bone matrix [33]. Fibronectin acts as an organizer of collagen fibrillogenesis and plays a role in its localized retention [34], so for the conversion of the TG-PEG hydrogel into collagen and mineral rich bone like matrix, fibronectin deposition should precede osteogenic differentiation which we have shown.

Alkaline phosphatase is an early marker of osteogenesis but also expressed by embryonic stem cells [35] which might explain why also the control samples stained positive for it since amniotic fluid cells

have been reported to be at least broadly multipotent [9]. Another explanation would be that 3D culture alone is enough to turn AFC into osteoprogenitors expressing alkaline phosphatase. Gels exposed to BMP-2 in 3D expansion media did not differ from the controls in terms of calcium deposition which might be due to the lack of phosphate source or the presence of bFGF which has been reported to counteract the osteogenic effects of BMP-2 [36].

In this study the osteogenic pre-differentiation *in vitro* of the constructs implanted *in vivo* was conducted for 2 weeks with a dexamethasone containing media. It was not established whether longer duration of differentiation *in vitro* would lead to improved bone formation *in vivo* but it has previously been shown that 2 weeks in osteogenic medium results in AFC commitment towards the osteogenic lineage with better *in vivo* performance compared to fully differentiated, AFC derived osteocytes [37]. Dexamethasone containing medium was chosen over equally well performing soluble BMP-2 as it was the cheaper option. However, in order to further enhance or accelerate bone formation *in vivo* slow release of BMP-2 immobilized in the TG-PEG hydrogels via biotin-streptavidin linker [38] could be used along with TG-tagged VEGF-165 [19] for long term promotion of vascularization.

The process of producing pre-vascularized constructs described here takes approximately 7 weeks from obtaining a sample of amniotic fluid at 15-18 weeks of pregnancy to having it ready for implantation (including isolating and expanding of AFC in 2D, testing them for viral infections, formation of 3D constructs and differentiating them). Thus, the described approach would be clinically feasible time wise. Next steps towards clinical application will include producing molds for making larger constructs featuring multiple channels for enhanced vascularization of the whole implant. In order to increase the stability of the vascular structures within the osteogenic constructs and to introduce a fresh source of stem/progenitor cells, the perfusion of undifferentiated AFCs into the osteogenic constructs along with the enAFC will be explored.

CONCLUSION

In conclusion, we have created and characterized both osteogenic constructs leading to bone formation *in vivo* and pre-vascularized constructs integrating with the host using amniotic fluid as a single cell source.

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Supplementary information

Materials and methods

Colony forming efficiency

Capability of AFC to form colonies from single cells after some time in culture was tested by plating them at clonal density. Cells at passage 5 from two donors were plated in 6-well plates at density of 100 cells per well. After 10 days the cultures were fixed and stained with 0.5% crystal violet (Sigma) in 20% methanol for 1 hour in room temperature. After two washes with tap water colonies of more than 50 cells were counted.

Characterization by FACS

Immunophenotypic analyses were performed on amniotic fluid cells (AFCs) after expansion at passage 5. Cells were collected, washed, resuspended in 100 µl PBS with 1mM EDTA and stained with mouse monoclonal anti-CD29 (Biolegend, San Diego, CA, USA), mouse monoclonal anti-CD34-PE (Miltenyi Biotec, Bergisch-Gladbach, Germany), mouse monoclonal anti-CD45-FITC (Miltenyi Biotec), mouse monoclonal anti-CD44-FITC (BD Pharmingen, Palo Alto, CA, USA), mouse monoclonal anti-CD73-PE (BD Pharmingen), mouse monoclonal anti-CD90-APC (BD Pharmingen), mouse monoclonal anti-CD105 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-CD49d-PE (Biolegend), mouse monoclonal anti-CD117-APC (Biolegend), mouse monoclonal anti-CD140α (Biolegend), mouse monoclonal anti-CD166-PE (BD Pharmingen), monoclonal anti-CD271-PE (Miltenyi Biotec), mouse monoclonal anti-NG2 (Abcam), mouse monoclonal anti-CD58-FITC (BD Pharmingen), or mouse monoclonal anti-CD146 (Chemicon) antibodies for 30 min. Samples which were not directly incubated with fluorochrome conjugated antibodies were washed and stained with DyLight™ 488 Goat anti-mouse IgG (Biolegend) for 30 min. After staining, cells were washed and fixed. Samples were analyzed using the BD FACS-Canto II and DIVA software, recording at least 10 000 events per marker. Comparative analysis was performed with FlowJo Version 7.6.4 (Tree Star, Inc., Ashland, OR, USA). Appropriate isotype controls from manufacturers were used as negative controls.

Immunostainings for evaluating endothelial differentiation

Samples were fixed with 4% paraformaldehyde for 20 min followed by 5 min incubation in 0.1 M glycine and two washes with PBS after which they were permeabilized and blocked with 1% BSA and 0.2% Triton X-100 in PBS for 60 min before staining For immunostainings, following primary antibodies were used at 1:100: mouse monoclonal anti-VEGFR2 (R&D Biosystems), mouse monoclonal

anti-CD31 (Millipore), rat monoclonal anti-Dll4, mouse monoclonal anti-Notch 1 (Biolegend), mouse monoclonal anti-integrin $\alpha_v\beta3$ (Chemicon) and FITC-conjugated polyclonal sheep anti-vWf. DyLight™ 488 Goat anti-mouse IgG (Biolegend) or Alexa Fluor 546 Goat anti-rat (Molecular Probes) were used as secondary antibodies at 1:200 when needed. After washing the samples three times in PBS, cell nuclei were stained with 5 ng/mL DAPI (40,6-diamidino-2-phenylindole) (Sigma Aldrich) in PBS for 10 min at room temperature. Sample evaluation was performed by epifluorescence microscope (BM550B, Leica Microsystems, Germany).

Results

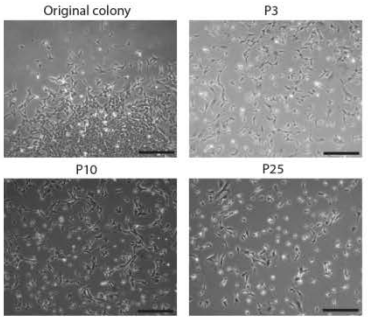
Characterization of AFCs

AFCs were cultured up to passage 25 with the morphology remaining unchanged (Supplementary figure 4.1A). Cells from passage 5 were able to form rapidly growing colonies at clonal densities (Supplementary figure 1B) displaying that self-renewal potential had not been lost in culture.

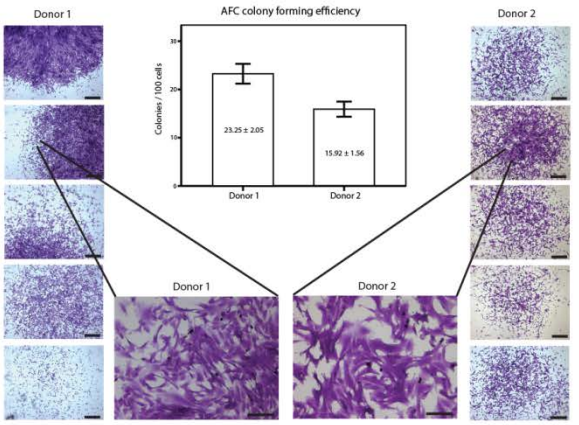
Amnion mesenchymal cells were also positive for CD44, CD73, CD90 and CD105 from the standard mesenchymal markers and negative for CD45 and CD34 excluding hematopoietic and endothelial contamination (Supplementary Figure 4.1B). They also expressed CD29, CD140α, CD146 and CD166 (Supplementary Figure 4.1C) associated with mesenchymal stem cells from various tissues.

AFCs could be differentiated towards osteogenic and endothelial lineages in 2D. Osteogenic differentiation was verified by stainings for alkaline phosphatase (after 10 days) and early initiation of calcium deposition (2-7 days, later time points not shown) (Supplementary Figure 4.2B). After 10 days of endothelial differentiation, AFCs displayed a change to a more cuboidal morphology and expression of proteins typical for endothelial cells (Supplementary Figure 4.2B).

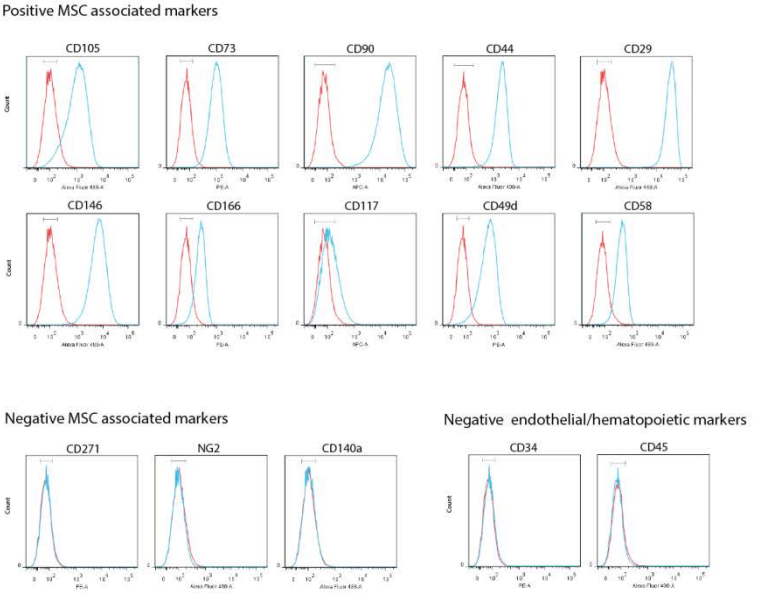
A


Original colony
P3
P10
P25

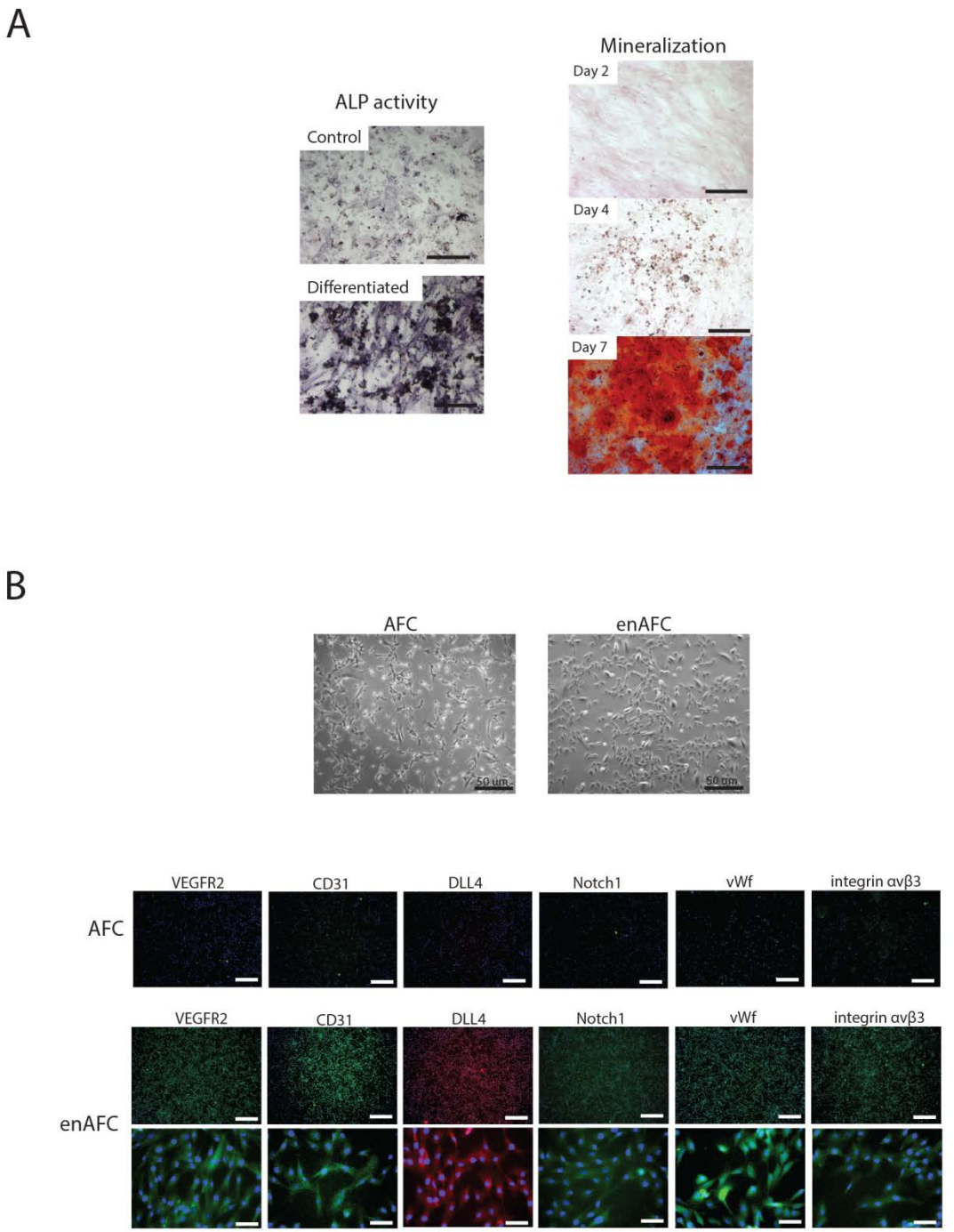
B


Donor 1
AFC colony forming efficiency
Donor 2
Colonies / 100 cells
23.25 ± 2.05
15.92 ± 1.56
Donor 1
Donor 2
Donor 1
Donor 2

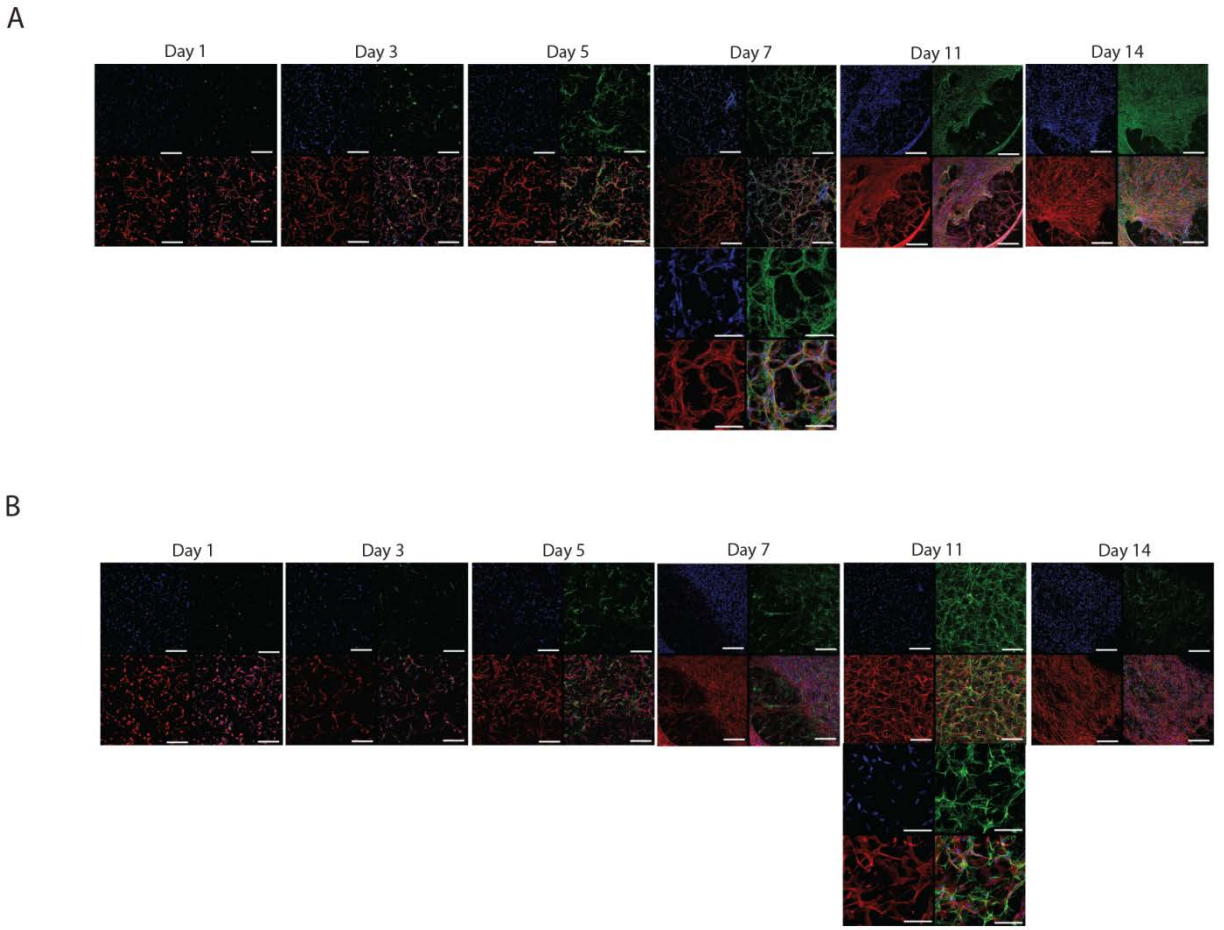
C


Positive MSC associated markers
CD105
CD73
CD90
CD44
CD29
CD146
CD166
CD117
CD49d
CD58
Negative MSC associated markers
CD271
NG2
CD140a
Negative endothelial/hematopoietic markers
CD34
CD45

Supplementary figure 4.1 Characterization of AFC. (A) Morphology over passages 5-25. (B) Subcloning efficacy of AFCs from two donors at passage 5, colonies stained with crystal violet. (C) FACS analysis of AFCs at passage 5 stained against characteristic mesenchymal (CD90, CD73, CD44, CD105, CD29, CD146, CD166, CD58 and CD140α) endothelial (CD34) and hematopoietic markers (CD45 and CD34).



Supplementary figure 4.2 Differentiation of AFC (A) Alkaline phosphatase activity after 10 days of osteogenic differentiation. Time series on mineralization during osteogenic differentiation indicated by alizarin red s staining. Scale bars 200 µm (B) Endothelial differentiation. Morphology after 10 days of endothelial differentiation in EGM-2 with additional 50ng/ml VEGF-165 and stainings for endothelial markers.



Supplementary figure 4.3 Time series of ECM production by AFC in TG-PEG hydrogels (A) Fibronectin (B) Collagen I. FN/Col1 green, F-actin red and Hoechst blue. Scale bars, 200 µm (low magnification) and 100 µm (high magnification).

CHAPTER 5 Conclusion

GENERAL DISCUSSION

The overall aim of this thesis was to tackle clinically relevant prenatal conditions by means of tissue engineering. The focus was to establish cell instructive environments with organized cellular arrangement and biochemical signals by using sophisticated biomimetic hydrogel materials. In the first chapter naturally derived scaffolds were used together with different gluing materials to seal and heal fetoscopy induced defects in fetal membranes *in vivo*. In the second chapter a more systematic *in vitro* approach was taken, using TG-PEG hydrogels for 3D cell culture, to find out which signals induce cellular processes vital for tissue regeneration in fetal membrane cells. In the third chapter the same biomimetic hydrogel system was employed to produce structured bone like constructs with vascular structures by controlled spatial organization of differentiated amniotic fluid cells for congenital skeletal defects. These examples demonstrate how an advanced biomaterials platform and clinically relevant sources of primary stem/progenitors can be combined to address reconstruction of tissues prenatally.

Applying native decellularized scaffolds and tissue glues for fetal membrane healing

Using a decellularized amnion plug containing all the signals from the native ECM was envisioned as a plugging strategy able to provide both initial sealing as well as a provisional scaffold for the initiation of healing and result in successful short and long term repair of punctured fetal membranes. Our in vivo data indicated that cells from the surrounding membranes could migrate along the scaffolds surface and to some extend enter inside the scaffold. Nevertheless, the amount of cells needed to initiate tissue repair processes using the scaffold would in reality be much higher than observed in these experiments. Thus, we concluded that moving to a more controllable in vitro approach would be needed in order to pin point the signals essentials for recruiting resident tissue cells and inducing their proliferation and ECM production. In general, the approach of using a plug together with the mussel mimetic glue as an initial immobilizing agent was deemed feasible and could be employed in future experiments with engineered next generation plugging materials.

Identifying novel growth factors affecting amnion membrane cells

Previous attempts to stimulate amnion cells have relied on traditional 2D cell culture approaches in which the cells' inherent ability to migrate and proliferate on plastic may have masked effects due to added growth factors. Using a 3D *in vitro* screening approach based on microtissues created from isolated cells we discovered three factors, namely PDGF, bFGF and EGF, that could recruit cells out of these tissue mimics. From this model we moved on to validating the results in an ex vivo recruitment model in which pieces of intact tissue were used instead of microtissues more realistically

recapitulating the situation *in vivo*. The previously identified growth factors could elicit similar migratory responses in this setup verifying their usefulness.

Engineering bone and vascular structures from amniotic fluid cells

In chapter 4 we reported the formation of next generation three dimensionally (3D) structured and vascularized bone tissue replacements, engineered from patient's own, phenotypically characterized and differentiated stem cells. Functional integration of these constructs was demonstrated in ectopic model *in vivo*. Evaluating the usefulness of these constructs for bone healing in a mouse calvarial defect model is currently underway. These experiments are done on adult mice and next step of of this project could be to implant the constructs in young pups orthotopically. It might also be of interest to test this tissue engineering approach in an actual *in vivo* model for cleft palate such as the TGF-β knock out mice [1].

FUTURE DIRECTIONS

Ex vivo fetal membrane healing models

As a first step towards establishing the ex vivo amnion healing model, the capability of amnion cells to migrate out of the amnion tissue into the synthetic TG-PEG hydrogel was investigated in chapter 2. Small pieces of amnion were encapsulated in hydrogels and within 4-9 days single cells began to migrate out of the tissue. Cell migration out of the tissue could be stimulated by growth factors enhancing migration *in vitro* (PDGF, bFGF and EGF) and unstimulated cultures did not result in cell outgrowth.

More realistic fetal membrane healing models would comprise of a combination of a thicker stromal layer with a layer of epithelial cells. To produce tissue engineered fetal membranes in a high throughput fashion, constructs could be assembled by layer-by-layer deposition or by 3D printing as done previously using TG-PEG hydrogels [2]. After maturation in culture such artificial membranes along side with amnion explants could be punctured and the regeneration capacity of growth factor presenting plugs evaluated in a more pertinent model.

The natural state of the cells in the amnion/fetal membranes during pregnancy is stretched both statically and cyclically. TG-PEG hydrogels used in chapter 2 are soft materials, so in order to create a more realistic model for membrane healing, the mechanical loading component is essential. In order to have more accurate and physiologically relevant information on the behavior of engineered matrices *in vitro*, a device allowing the culture of membrane explants and tissue engineered membrane analogs under dynamic stretching conditions would be required. The design of such a setup could be based on an inflation device previously developed in our lab, which allows the biaxial testing of membrane mechanical properties under physiological pressures by inflating fixed membranes with water [3]. A miniaturized cell culture compatible version of the system could integrate both culture and mechanical

testing of regenerated membranes and determined signals or combinations of signals which best promote tissue healing.

In vivo evaluation of cell instructive matrices with growth factor reservoirs

To give our findings clinical relevance in treating iPPROM, optimized healing inducing matrices from chapter 3 will be further tested *in vivo*. Initial tests will be performed using the mid-gestational rabbit model described above. However, because of the short duration of rabbit gestation and differences in the attachment of the fetal membranes to the uterine wall compared to humans, final *in vivo* investigations could be done in sheep. The ovine model would allow long term observation of material stability and tissue regeneration as well as testing plug application strategies in a way better translatable to humans.

The most promising factors will be produced and recombinantly expressed as matrix binding factors and the *in vitro* activity of such factors will be evaluated using migration and proliferation assays described in chapter 3. In such a microenvironment the growth factors will only be released when cells proteolytically degrade the matrix. TG-tagged versions of all three factors have been produced and initial tests using TG-PEG plugs containing matrix bound PDGF have been initiated in the mid-gestational rabbit model.

Spatiotemporal delivery of bound growth factors for regenerating bone with vascular structures

Bone morphogenic proteins (BMPs) are most prominent growth factors involved in bone regeneration and applied in clinical setting in treatment of orthopedic applications such as spinal fusion, non-unions, and oral surgery [4]. They are not only osteoinductive but also induce angiogenesis by upregulating expression of vascular endothelial growth factor (VEGF) [5] which again further enhances bone formation. Despite the pre-clinical and clinical successes of BMP-2 treatment, there have been reports on severe side effects due to the extremely high concentrations of soluble growth factor used to achieve the desired bone healing. As described in chapter 1, several strategies exist to tether growth factors and other bioactive moieties to synthetic and naturally derived hydrogels which restrict the growth factors closer to the defect site and slow down the rate of release allowing the use of lower growth factor concentrations. There are several peptide linkers with different affinities and degradability dependent release kinectics available and being developed for the TG-PEG system. Thus, dual growth factor delivery techniques with specific temporal release profiles could be applied together with the AFCs to mimic the natural sequence of bone development and repair.

In chapter 4 chemical differentiation media was chosen for osteogenic predifferentiation of implanted constructs over growth factor based approach for cost purposes, but BMP-2 mediated differentiation of AFCs was also show *in vitro*. To further enhanced the bone healing observed using only predifferentition *in vitro*, BMP-2 could be immobilized in the constructs for continued osteogenic

stimulation of the AFC and resident cells *in vivo*. Dual delivery of BMP-2 and VEGF has been shown to improve bone regeneration *in vivo* [6, 7] and it would be possible to incorporate VEGF alongside BMP-2 in the bulk of the TG-PEG hydrogel for later release to promote vascular ingrowth. Perfusion of endothelial cells into the channel prior to implantation could also be done by embedding them in a hydrogel with additional bound VEGF. Additional factors promoting bone regeneration and angiogenesis such as bFGF and PDGF could also be added for ultimate fine tuning of the coexistence of osteogenic and vascular structures.

Clonal analysis of amniotic fluid cells

Amniotic fluid contain a myriad of different cell types originating from the fetus and the fetal membranes. We thus hypothesize that within the amniotic fluid not only cells with different origin but also with different differentiation status and potential can be found. However, as little is known about the true potential of individual freshly isolated AFCs since in most tissue engineering approaches heterogeneous bulk AFCs or cell lines derived from a single clone were employed. Most published studies have sought after the most primitive cells in amniotic fluid in hopes of having in their hands something corresponding to embryonic stem cells in their potency, but being ethically uncontroversial. Of course a pluripotent cell offers the most flexibility in terms of differentiation options and tissue engineering applicability in theory but identifying more committed tissue specific progenitor cells might be more simple and straightforward in practice.

If proliferation and expression of certain markers associated with pluri- or multipotency or specific progenitor cells such as Oct-4, CD117, CD146, NG2 or CD34 correlate with differentiation capabilities of amniotic fluid cells at clonal level they could be used as a criteria for selecting cells for different tissue engineering purposes. Most common media compositions used in the literature include supplementation with 15-20% FBS, 5 ng/ml bFGF or so called Chang supplements and would also be worth investigating whether there are differences rising from cell isolation and culture in different media at clonal level.

The heterogeneity of AFCs should be characterized at a single cell level either by using limited dilutions on a traditional well plate or by employing PDMS microwells [8] for a more sophisticated and high throughput approach. As micro-wells can be formed with different stiffness and specifically coated with any protein of choice, the proliferative potential of a large number of individual cells in response to mechanical properties and extracellular matrix proteins can be followed by time-lapse microscopy. As expected, our initial data indicate that both proliferation kinetics and morphology of cells which have been isolated based on described procedures are largely heterogeneous. Hundreds of clones with variable properties can be generated from a 1 ml sample of amniotic fluid (our unpublished data) and by evaluation of proliferation, phenotype and differentiation potential of the whole population versus these clones more light could be shed on the cellular hierarchy and tissue engineering potential of amniotic fluid.

Engineering other fetal tissues

In this thesis amniotic fluid cells were used for engineering vascularized bone but the concept of spatiotemporal structuring of biomimetic hydrogels together with controlled differentiation of these multipotent cells could be employed to produce other fetal tissues as well. For example amniotic fluid cell seeded extracellular matrix scaffolds have already been tried for the treatment of congenital diaphragmatic hernia in sheep with results comparative to standard acellular materials [9]. We believe it would be possible to improve the implantable grafts by using a defined culture environment, differentiating the 3D organized cells towards tenogenic lineage while subjecting the constructs to mechanical loading during maturation and finally adding prevascularization for viability of larger constructs.

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